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The *rough deal* protein is a new kinetochore component required for accurate chromosome segregation in *Drosophila*

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SUMMARY

Mutations in the *rough deal* (*rod*) gene of *Drosophila* greatly increase the missegregation of sister chromatids during mitosis, suggesting a role for this gene product in spindle or kinetochore function. The activity provided by *rod* also appears to be necessary for the recruitment of two known kinetochore components, Zw10 and cytoplasmic dynein. In this paper we describe the cloning of *rough deal* and an initial cytological characterization of its product. The Rod protein shares no identifiable structural motif with other known proteins, although apparent homologs exist in the genomes of nematode and man. By immunocytochemistry we show that Rod displays a dynamic intracellular staining pattern, localizing first to kinetochores in prometaphase, but moving to kinetochore microtubules at metaphase. Early in anaphase the protein is once again restricted to the kinetochores, where it persists until the end of telophase. This behavior is in all respects similar to that described for Zw10, and suggests that the proteins function together.

Key words: Mitosis, Anaphase, Spindle

INTRODUCTION

The kinetochores are the sites of microtubule (MT) attachment to eukaryotic chromosomes (Pluta et al., 1995; Rieder and Salmon, 1998). In addition to the molecular motors required for chromosome congression to the metaphase plate and for chromatid movement to the poles at anaphase, kinetochores also contain proteins whose function is to assure that chromosomes segregate to daughter cells with a very high degree of fidelity. Among these are major components of a checkpoint apparatus which controls the timing of the metaphase-anaphase transition. This checkpoint goes by many names (see discussion in Rieder and Salmon (1998), but we will refer to it as the spindle assembly checkpoint (SAC). The SAC monitors events that occur at the kinetochore to verify that all the chromosomes are properly oriented on the mitotic spindle before anaphase is allowed to begin (reviewed by Elledge, 1998; Rieder and Salmon, 1998; Wells, 1996).

In *Drosophila*, two gene products have been identified whose activities are apparently required for the concerted release or movements of sister chromatids (during mitosis) or of chromosome homologs (during the first meiotic division) (Karess and Glover, 1989; Williams et al., 1992), but which do not correspond to known components of the SAC. In cells mutant for the genes *rough deal* (*rod*) (Karess and Glover, 1989) or *zw10* (Williams et al., 1992), at least some components of the SAC appear to be functioning correctly (Basu et al., 1998) but one finds abnormal anaphases in which the majority of chromatids (or chromosomes in meiosis I) have moved to the poles, while one or more chromatids or chromosomes lag behind, remaining in the vicinity of the metaphase plate. This leads to high levels of aneuploidy and often to the death of the individual.

The *zw10* gene has been shown to encode an 85 kDa component of the kinetochore (Williams et al., 1992), which during the course of mitosis changes its location in an unusual and intriguing manner (Williams et al., 1996; Williams and Goldberg, 1994; Williams et al., 1992). It is first found on kinetochores at prometaphase, but once the chromosome is properly bi-oriented on the spindle, Zw10 is found distributed irregularly along the kinetochore microtubule (K-MT) fibers. At the onset of anaphase, Zw10 is once again found exclusively on the kinetochores, where it remains until the end of telophase. The behavior of Zw10 suggests that it is sensitive to the physical tension across the kinetochore, which is believed to be a major indicator of proper chromosome biorientation on

the spindle (Li and Nicklas, 1995). Despite the presence of Zw10 on the mitotic apparatus during prometaphase and metaphase, the congression of chromosomes to the metaphase plate appears to be independent of Zw10 activity. Only during anaphase do *zw10* null mutants display abnormalities.

Null mutations in the *rough deal* (*rod*) gene cause a phenotype essentially indistinguishable from that of *zw10*: elevated levels of aneuploidy resulting from failure of coordinated sister chromatid separation, and no other obvious phenotype prior to anaphase onset. In fact, it has been shown that Zw10 fails to localize to kinetochores or K-MTs in *rod* mutants, showing that the Rod protein is actually required for the proper functioning of Zw10 (Williams and Goldberg, 1994). More recently, Starr et al. (1998) have shown that the activities of *zw10* and *rod* are each required for the recruitment of cytoplasmic dynein to the kinetochore, which suggests that at least part of the *rod* and *zw10* mutant phenotypes may be attributable to the absence of this microtubule motor at the kinetochore

In this paper we describe the cloning of *rough deal* and an initial characterization of the encoded protein's behavior during mitosis and male meiosis. We find that *rod* encodes a 240 kDa protein with no structural similarity to other known proteins, though related proteins are encoded by the genomes of nematodes and humans. During the mitotic cycle, Rod protein associates with kinetochores and K-MTs in a dynamic manner similar to that described for Zw10, lending support to the idea that these two proteins act together in assuring accurate chromosome segregation.

MATERIALS AND METHODS

Fly stocks

The original description of *rough deal* alleles $rod^{H4.8}$, rod^{X1}, rod^{X2}, rod^{X3}, and rod^{X4} can be found by Karess and Glover (1989). The alleles rod^{X5} and rod^{X6} were found subsequently (unpublished). The alleles rod^{X1} – rod^{X6} were all identified in an X-ray mutagenesis screen as described by Karess and Glover (1989). The alleles $rod^{H4.8}$, rod^{RP153} and rod^{29D} found in three independent P-M hybrid dysgenesis mutagenesis screens in three different laboratories. The latter two were kind gifts from M. Gatti (Rome) and M. Goldberg (Ithaca), respectively. Despite their origins, none contained a P element at the *rod* locus. All other stocks mentioned were obtained from the Bloomington (Indiana, USA) *Drosophila* stock center. Other genetic markers are described by Lindsley and Zimm (1992). The *rod* mutant chromosomes, marked with *ca*, are maintained balanced over *TM6*, carrying the dominant larval marker *Tubby* (*Tb*). Homozygous *rod* mutant larvae can therefore be distinguished as Tb^+, while their heterozygous siblings are *Tb*. Flies were raised on standard medium at 25°C.

Cytogenetic localization of the *rough deal* gene

Available deficiencies and duplications at the tip of chromosome 3R delimited the *rod* gene to the cytogenetic interval 100C1,2-100D1,2 (Karess and Glover, 1989). However, no useful extant deficiencies uncovered the mutation. In order to obtain a useful deficiency and to more accurately map the location of *rod*, we took advantage of the presence of the *awd* gene at 100D1,2 whose dominant conditional lethal allele $awd^{K\text{-}pn}$ is lethal in the presence of the *pn* mutation on the *X* chromosome (Biggs et al., 1988; Sturtevant, 1955). We undertook an X-ray mutagenesis, looking for viable flies of genotype *pn;* $awd^{K\text{-}pn*}$ where * would be a potential deletion around the *awd* locus. For several reasons, we considered it possible that *rod* might be closely linked to a haplo-lethal locus, or might even be haplo-lethal itself; (i) the lack of extant deficiencies uncovering *rod*; (ii) the absence of cytologically-observable aberrations associated with any of 6 independent X-ray induced *rod* alleles; (i) the fact that at least one *Minute* was known to reside distal to 100C (see Kongsuwan et al., 1986). To provide an extra wild-type copy of any potentially haplo-insufficient genes in the region, the screen was performed with flies containing *Dp (3;1)1A*, an *X* chromosome carrying the 100B5,7-Telomere region of chromosome-*3* (Kongsuwan et al., 1986) ($awd^{K\text{-}pn}$ is dominant lethal in combination with *pn* even in the presence of two wild-type copies of *awd*). Male flies homozygous for *ca* $awd^{K\text{-}pn}$ were irradiated with 1000 rads from a ^{60}cobalt source and crossed to females homozgyous for *pn Dp(3;1)1A*. The *pn Dp(3;1)1A,* awd^+*/Y; ca* $awd^{K\text{-}pn}$/+ males should die from the lethal interaction of *K-pn* and *pn*, unless a mutation or deletion of $awd^{K\text{-}pn}$ allowed such males to survive. From approximately 14,000 mutagenized chromosomes, one surviving male was isolated with the following properties: (i) it had sustained a deletion called *Df(3R)P4.1* from 100C4,5 to 100E, as well as a pericentric inversion, breaking at 62B and 100C4,5; (ii) it is haplo-lethal, that is, *Df(3R)P4.1 /+* heterozygotes die at the late pupal stage in the absence of the covering duplication *Dp (3;1)*1A, however, larval neuroblasts of the deficiency heterozygotes show no aneuploidy, that is they do not have the *rod* phenotype; (iii) rare *Df(3R)P4.1/+* escapers (<1% of expected offspring) have a *Minute* phenotype; (iv) *Df(3R)P4.1/rod* larvae do have the *rod* mutant phenotype, with a high frequency of abnormal anaphases and aneuploid cells. This deletion therefore confirmed the cytogenetic location of *rod*, restricting it to the interval 100C4,5-100D1,2, a region of approximately 250 kb (Spierer et al., 1983). The absence of a *rod* (aneuploidy) phenotype in the *Df(3R)P4.1/+* genetically *Minute* larvae, and the absence of a *Minute* phenotype in heterozygotes of *rod* protein null mutants (see Results), demonstrates that *rod* itself is not haplo-insufficient, and therefore is not likely to be causing the observed *Minute* phenotype.

Molecular cloning of *rough deal*

To initiate a chromosome walk in the interval 100C4,5-100D1,2, cloned DNA fragments of unique sequence DNA were obtained by microdissection and microcloning (Scalenghe et al., 1981) of the 100C4,5 region. Briefly, 100C4,5 polytene bands from a dozen wild-type polytene chromosomes were scraped with a microneedle into a hanging microdrop of buffer, the DNA extracted, digested with *Eco*RI and ligated to lambda vector NM522, packaged and plated. From this operation about 200 clones were obtained. Ten were chosen at random and used for in situ hybridization to wild-type polytene chromosomes. Three of these contained single copy sequence that hybridized to 100C4,5 and were therefore used as the initial probes to screen a *Drosophila* cosmid library (Tamkun et al., 1992, gift of J. Tamkun, U. C. Santa Cruz, USA). Restriction fragments from the ends of the cloned inserts of the cosmids were used to extend the walk in both directions from the entry points. Fragments representing the entire insert of each isolated cosmid were used as probes on Southern blots of digests of genomic wild-type and heterozygous *rod* mutant DNA, in order to identify polymorphisms associated with one or more of 7 mutant *rod* alleles (rod^{X1} through rod^{X6} and the original allele $rod^{H4.8}$). Also included on the blot was DNA of *pn Dp(3;1)1A; Df(3R)P4.1/TM6*, with the cytological break in the 100C4,5 region. After examining approximately 250 kb in two contigs, a cosmid in one contig was identified that detected DNA rearrangements associated with this deficiency, indicating that it had crossed the breakpoint. In situ hybridization of the cosmids to polytene chromosome spreads heterozygous for the deficiency allowed the direction of the walk relative to the centromere to be determined. Approximately 30 kb centromere-distal from the *Df(3R)P4.1* breakpoint, DNA from three of the mutant *rod* alleles displayed polymorphisms when probed with a cosmid, called pC3L9 (see Fig. 1). This cosmid was

subcloned for further examination, and relevant portions were sequenced.

The structures of the mutant alleles rod^{X1} and rod^{X5} were determined by sequencing of PCR-generated fragments spanning the lesions visible by Southern blot. For the insertion mutation $rod^{H4.8}$ large PCR fragments spanning the insertion were obtained by using Klentaq (Clontech) and TaqLong (Stratagene). The identity of the insertion element as well as its site of insertion was determined by sequencing of portions of the amplified fragments containing flanking *rod* sequences.

Germline transformation

The Tamkun cosmid library (Tamkun et al., 1992) was generated in the CosPer vector, in which the cloned material and a selectable w^+ genetic marker are flanked by the P element sequences required in cis for transposition. Cosmid pC3L9, containing 34 kb of *Drosophila* genome, was purified by Qiaprep columns (Qiagen), and mixed with the helper plasmid pUC hs Δ2-3 (Mullins et al., 1989), at a mass ratio of 10:1, and a final total concentration of 250 μg/ml, and injected into *y* w^{67} *Drosophila* eggs using standard techniques (Ashburner, 1989). The single w^+ transformant isolated contained a homozygous viable transgene on chromosome-*2*. A stock was constructed that contained the homozygous transgene as well as the two chromosome-*3* balancers *TM3, Ser/TM6, Tb*. To test for rescue of the *rod* mutants by the *C3L9* transgene, *w; P[*w^+*, C3L9]; TM3/TM6* flies were crossed to *w; rod/TM6*. Offspring carrying the transgene and the *rod* allele balanced over *TM6* were identified and intercrossed. Among the progeny of this latter class, homozygous *rod* flies were identified as being Tb^+, and the number of copies of the *C3L9* transgene determined by eye color (white, none; pink, one; red, two). To assess the rescue of the *rod* mitotic phenotype, flies homozygous for the transgene and for a given *rod* allele were crossed to *rod/TM6*. All non-Tb larvae should then be homozgyous *rod* and carry a single copy of the transgene. Neuroblasts of third instar brains from such larvae were then examined for abnormal mitotic figures. All the *rod* alleles could be rescued to healthy adulthood by a single copy of the transgene.

Molecular biology techniques

High density colony screening, plaque screening of cDNA libraries, subcloning, primer extension, southern and northern blotting all used standard protocols (Sambrook et al., 1989). Cosmid DNA was purified either by CsCl gradient centrifugation or by Qiaprep columns (Qiagen). Sequencing was by dideoxy chain-termination using the Sequenase kit (Amersham). Embryo and imaginal disc cDNA libraries were a gift from Nick Brown (Brown and Kafatos, 1988). The structure of the transcript was determined from analysis of cDNA clones and from cDNAs generated by reverse transcriptase followed by PCR amplification. For RT-PCR, total RNA was isolated (Chomczynski and Sacchi, 1987) from the *Drosophila* S2 cell line. Reverse transcription was performed using Superscript (Gibco-BRL), and primed either with oligo dT or with random hexamers. PCR was performed using primers based on the known sequence in genomic DNA of the region believed to encode the transcript. Oligonucleotides were synthesized by Eurogentec (Seraing, Belgium), or Isoprim (Toulouse, France). Primer extension was used to identify a likely 5′ end of the transcript.

Antibody production and immunocytochemistry

A 1.2 kb *Bam*HI-*Eco*RI fragment of a cDNA generated by RT-PCR and encoding amino acids 10-417 of the predicted Rod protein was ligated in frame to the His-6 tag of the pET 21c expression vector, and the protein expressed in BL21(DE3) cells after induction by 1 mM IPTG. Following His-6 affinity purification on a Ni affinity column (Novagen), approximately 300 mg of purified protein was sent for antibody production in rabbits (Eurogentec, Seraing, Belgium). The antibody, called BE40, was tested by immunoblotting against the immunogen and against fly cell extracts. Affinity purified antibodies were prepared by incubating the crude serum with strips of immunoblot containing the 40 kDa fusion protein, washed extensively, and eluted in 0.1 M glycine, as described (Harlow and Lane, 1982).

For staining larval neuroblasts, third instar homozygous *rod* mutant larvae were collected, the brains dissected and treated exactly as described by Williams and Goldberg (1994). Testes were dissected either from late third instar or early pupal stage individuals, and prepared as described (Williams et al., 1996). Both affinity-purified and crude serum were used for immunostaining, and gave identical results. Antibodies were used at the following dilutions: anti-Rod BE40, dilution 1/500; anti-α-tubulin monoclonal DM1A (Sigma), dilution 1/1000; Secondary antisera Texas Red anti-mouse IgG, FITC anti-rabbit IgG (Jackson Immunochemicals) dilution 1/50; Alexa 594 and 488 anti-mouse and anti-rabbit IgG (Molecular Probes), dilution 1/300. DNA was labeled with DAPI at 0.5 μg/ml.

Immunoblotting

Drosophila protein extracts for the immunoblots of Fig. 4 were prepared from third instar larval brains, dissected in 0.7% saline. The brains (10 per lane for wild-type and *C3L9* transgenic brains, 12 per lane for the generally smaller homozygous *rod* mutants,) were collected in a few microliters of saline in the lid of a chilled microtube and manually crushed with dissecting forceps. The tissue was incubated on ice for 5-10 minutes in the presence of 50 μg/ml DNAse I. Protein extracts of the *Drosophila* S2 cell line were prepared from exponentially growing cultures. Cells from a 25 cm^2 culture flask were harvested, rinsed once in phosphate buffered saline (PBS), resuspended in 0.5 ml of PBS, lysed by sonication, then incubated 10 minutes with DNAse I as above. One volume of standard Laemmli gel 2× sample buffer was added to the protein extracts and the samples were immediately boiled for 5 minutes. The tubes were briefly centrifuged to remove insoluble material. The samples could be stored at this point at –20°C for several weeks without any obvious change in the results obtained. Samples were resolved by SDS-polyacrylamide gel electrophoresis using a stacking gel and a 5% acrylamide resolving gel, and standard Laemmli solutions (Harlow and Lane, 1982), in a Bio-Rad Mini Protean II apparatus. Gels were run at 200 V for 1 hour. Following electrophoresis, the protein was transferred to Hybond-PVDF (Amersham) membrane by semi-dry electrotransfer (80 V for 1 hour) using a LKB-Bromma Novablot apparatus. The membrane was then stained briefly with Coomassie Blue to confirm the uniformity of the transfer, destained in 90% methanol, and blocked for 30 minutes with TBST-milk (20 mM Tris, 137 mM NaCl, pH 7.6, 0.1% Tween-20, 5% nonfat dry milk). The membrane was incubated with unpurified anti-Rod antibody (diluted 1/500) overnight at 4°C, washed extensively in TBST-milk, and incubated with peroxidase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch) at a dilution of 1/15,000 for 30 minutes at room temperature. After washing in TBST-milk the blot was developed using ECL chemiluminescent detection reagents (Amersham) and exposed to film, following manufacturer's protocol.

Cytology and microscopy

For simple phase-contrast observation of neuroblasts, brains of third instar larvae were dissected fixed and stained with aceto-orcein as described (Karess and Glover, 1989). Treatment with colchicine (10 μM) or taxol (10 μM) was for 30 minutes, followed by hypotonic shock in 1% Na Citrate for 5 minutes, and fixation as described (Karess and Glover, 1989). Cells were viewed with a ×63 objective using a Nikon Microphot microscope with phase contrast and epifluorescence. Images were collected with a Princeton Instruments cooled CCD camera, using GRAFTEK (France) Fluograb software. Adobe Photoshop was used for subsequent treatment of digital images.

RESULTS

Cloning of the *rough deal* locus

Cytogenetic analysis (see Materials and Methods) placed *rod* in chromosome region 100C4,5-100D1,2 near the tip of chromosome 3R. A chromosome walk was initiated in this cytogenetic interval by isolating overlapping cosmid clones which were used as probes on southern blots containing digestions of genomic DNA from a panel of *rod* mutants and from flies containing the deficiency *Df(3R) P4.1*, whose breakpoint at 100C4,5 defines the proximal limit of the region. Approximately 30 kb centromere-distal to this breakpoint, a cosmid insert was identified that detected DNA polymorphisms associated with three different *rod* alleles, rod^{X1} rod^{X5} and $rod^{H4.8,}$ all within a 5 kb region, making it likely that this cosmid contained all or part of the *rough deal* gene (see Fig. 1). This cosmid, called C3L9, was used directly for P element mediated germline transformation (see Materials and Methods). A single transformant was identified among 53 G1 lines. One copy of the C3L9 transgene was found to complement fully all the extant mutant alleles of *rod*, thus confirming that the *rod* gene in its entirety was included within it.

cDNA clones hybridizing immediately left and right of the polymorphic 5 kb region were identified by screening several cDNA libraries, and fell into two classes, named pNB20A (3.5 kb) and pNB5.2 (3.8 kb), after the longest cDNAs in each class. These two cDNAs did not overlap and were found to correspond to transcripts with opposite polarities, implying that they represent two different genes. Because of data indicating that pNB20A was an incomplete representation of a much longer mRNA, we isolated cDNA sequences corresponding to the remaining 5′-end of this transcript by RT-PCR, using primers based on the genomic sequence from the region between the two cDNA classes. We were subsequently able to assign pNB20A as the *rough deal* transcript because five *rod* alleles, including the 3 mentioned above, were eventually found to have lesions within the transcription unit (see below).

Structure of the *rough deal* gene

The structure of the *rod* gene as shown in Fig. 1 was determined by combining sequence information obtained from RT-PCR products and cDNA clones as well as from the corresponding genomic sequences within the C3L9 cosmid. The gene covers approximately 8.5 kb of the *Drosophila* genome, and is comprised of 14 exons and 13 small introns. The transcript itself is about 6.8 kb in length. A single open reading frame begins at an ATG at position 1228 (measured from the upstream genomic *Bam*HI site), and encodes a protein 2098 amino acids long, with a predicted molecular weight of 240 kDa. There is no evidence for alternatively spliced forms, at least in the tissues from which the cDNAs and RT-PCR clones were derived (imaginal discs and *Drosophila* S2 cells, respectively). A possible transcription start site is nucleotide 878, as determined by primer extension; this site is also just downstream from a TATA box. A candidate for the 5′ end of

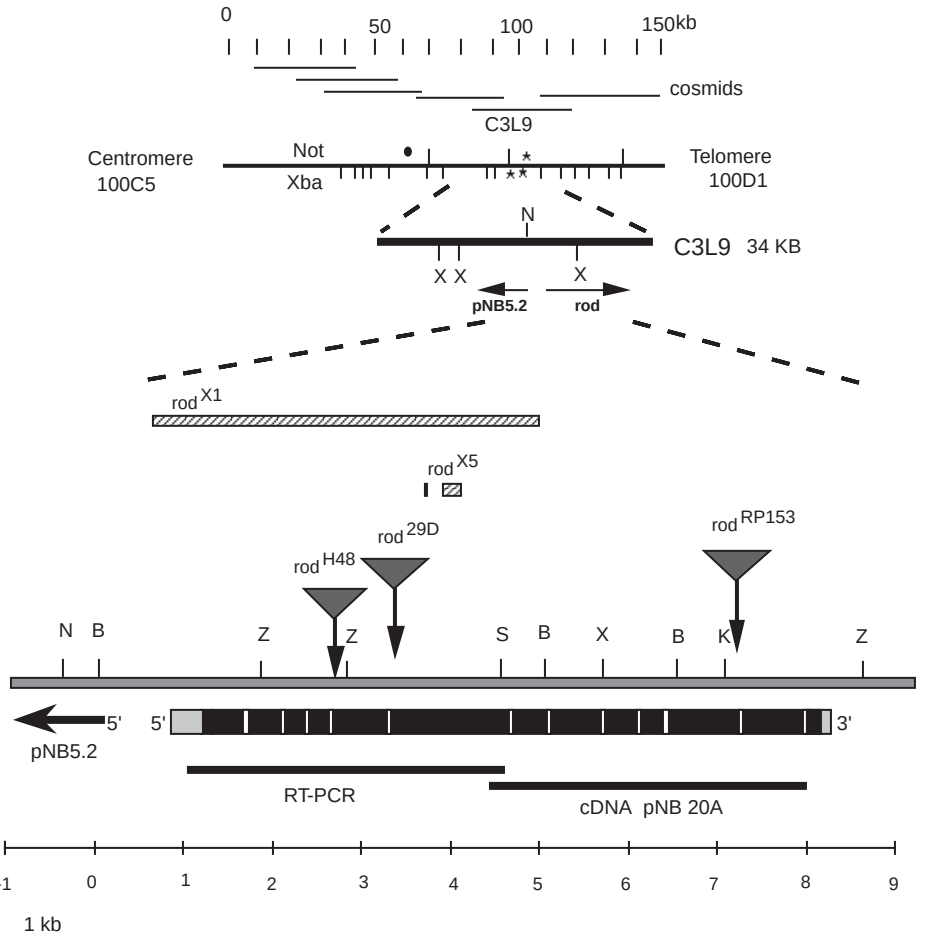

Fig. 1. Physical map of the *rough deal* region. Top: A portion of the cosmid walk in the 100C4,5 region of chromosome arm 3R, and a map of the chromosome region covered by the walk, indicating *Not*I and *Xba*I sites. The dot marks the position of the proximal (100C4,5) breakpoint in the deficiency *Df(3R)P4.1* (100C4,5; 100E). The stars (*) mark positions of DNA polymorphisms associated with mutations of *rod*. Cosmid C3L9 is shown below the map, showing the orientations and approximate extents of the two identified transcription units (pNB5.2 and *rod*). This cosmid contains the entire *rod* transcription unit and was used directly for germ-line transformation and rescue of the *rod* mutant phenotype. Bottom: The structure of the *rod* gene and transcript. Sites of DNA rearrangements associated with mutations of *rod* are indicated: deletions as hatched rectangles, and insertions as shaded triangles (the sizes of the insertions are not shown to scale). Introns are shown as white lines in the transcription unit, and noncoding 5′ and 3′ regions of the transcript are shown in gray. The coordinates are in kb, as measured from the *Bam*HI site (which is defined as the 0 position) just to the left of the beginning of the *rough deal* transcript.

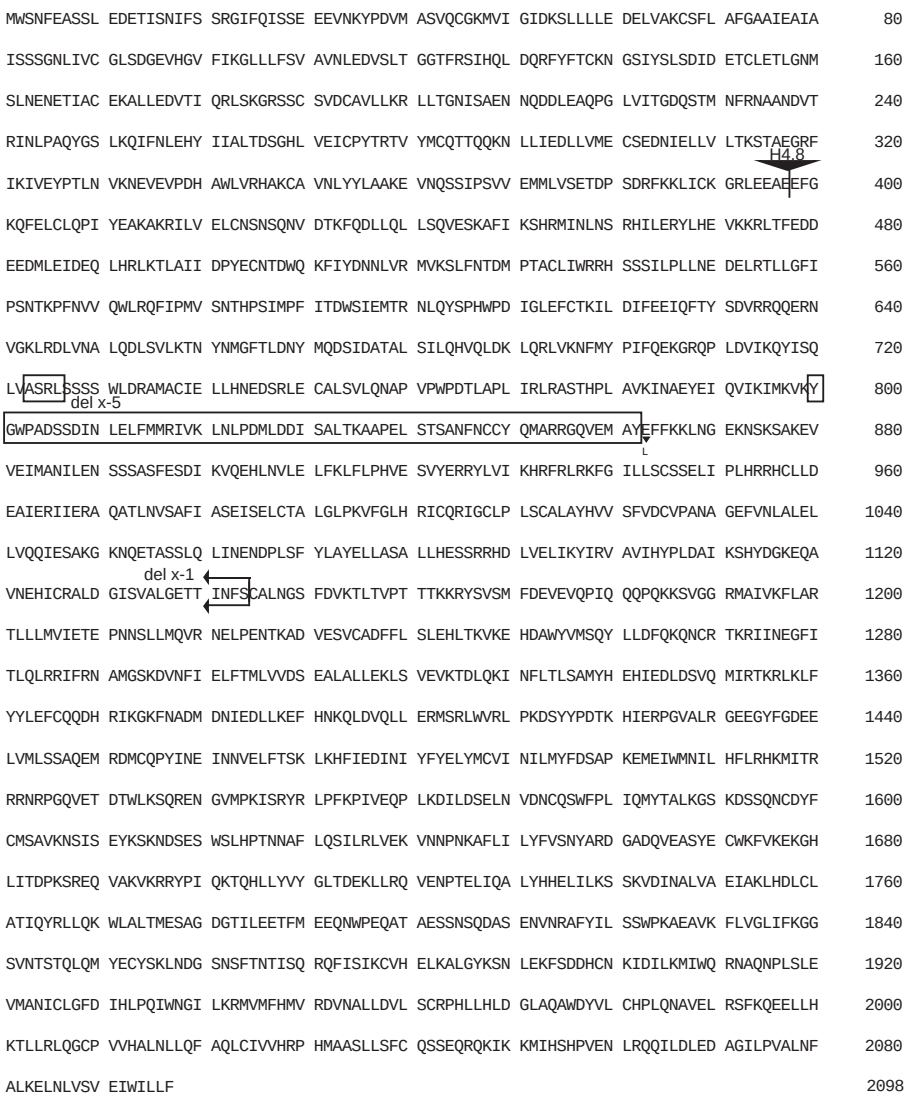

Fig. 2. The amino acid sequence of the *rough deal* protein. The downstream limit of the region deleted in rod^{X1} is indicted by a pair of arrows. The deletions in rod^{X5} are boxed, and the altered amino acid is written below the wild-type sequence. The site of the transposon insertion associated with $rod^{H4.8}$ is indicated by a triangle. The entire genomic sequence for the *Drosophila rough deal* locus has been deposited in GenBank (accession number AF114152).

the adjacent gene represented by pNB 5.2 (which is transcribed in the opposite sense) was identified in the database of the Berkeley *Drosophila* Genome Project/HHMI EST Project (unpublished). This EST (clone ID 21971) begins at nucleotide 509, leaving an approximately 370 bp AT-rich intergenic region between the two genes. No ESTs to the *rod* transcript were found.

Of the nine mutant alleles of *rod* examined, two (rod^{X1}, rod^{X5}) are deletions and three ($rod^{H4.8}$, rod^{RP153}, and rod^{29D}) are insertions of mobile DNA within the transcription unit. The remaining four (rod^{X2}, rod^{X3}, rod^{X4} and rod^{X6}), all isolated in an X-ray mutagenesis screen, have no lesions detectable by Southern blot. The three insertion mutations were isolated during three independent P element mutagenesis screens, yet none of the three carried a P element. In the case of $rod^{H4.8}$, a Doc retrotransposon had inserted in an intron following nucleotide 2730. rod^{RP153} contains a 5 kb insertion and rod^{29D} a 5.5 kb insertion, but the precise sequence at the site of the lesions in these two insertion alleles was not determined. The 4.6 kb deletion in rod^{X1} removes nucleotides 538-5109, essentially deleting the 5′ half of the *rod* gene up to codon 1144 as well as a portion of the 5′ intergenic region between *rod* and the transcription unit represented by pNB5.2. rod^{X5} appears to have sustained several changes within the 500 bp sequenced: a deletion of 12 bp and another of 189 bp, and a substitution of 3 new nucleotide pairs at the end of this deletion; all these changes retain the open reading frame.

The sequence of the Rough Deal protein

The amino acid sequence of the 240 kDa Rod protein is presented in Fig. 2. Although it contains no recognizable protein sequence motifs, a BLAST search (Altschul et al., 1997; Gish and States, 1993) of various databases identified genes of unknown function with significant sequence similarity in the genomes of humans and nematodes. A CLUSTAL-W alignment (Thompson et al., 1994) of these three sequences shown in Fig. 3. The sequence identity among the fly, worm and human Rod homologs is around 20%, and the overall similarity is 40%. The regions of sequence similarity are broadly distributed throughout the protein but are strongest in the amino-terminal third, which is predicted to be largely α-helical, and the carboxy-terminal fifth. The region from residues 368-438 of the *Drosophila* Rod protein is among the most highly conserved: sequence identity with either the human or worm homologue is around 50%, with similarity approaching 76%. It may be significant that no sequence related to Rod could be identified in the complete genome of the yeast *S. cerevisae* (see Discussion).

Expression of Rod protein

A rabbit polyclonal antibody was raised against the N-terminal 40 kDa of the *Drosophila* Rod protein (see Materials and Methods). On western blots the antibody detects a protein near the expected size (ca. 200 kDa) in *Drosophila* S2 tissue culture cells and in wild-type third instar larval brains (Fig. 4). Although other proteins are visible on the western blots, these are apparently unrelated to Rod, as they are present in unchanging relative amounts in all the fly genotypes examined, whereas the 200 kDa protein was more abundant in the *C3L9* transgenic line (compared to wild type) and absent or greatly reduced in all of the mutant lines. Eight of the nine mutant *rod*

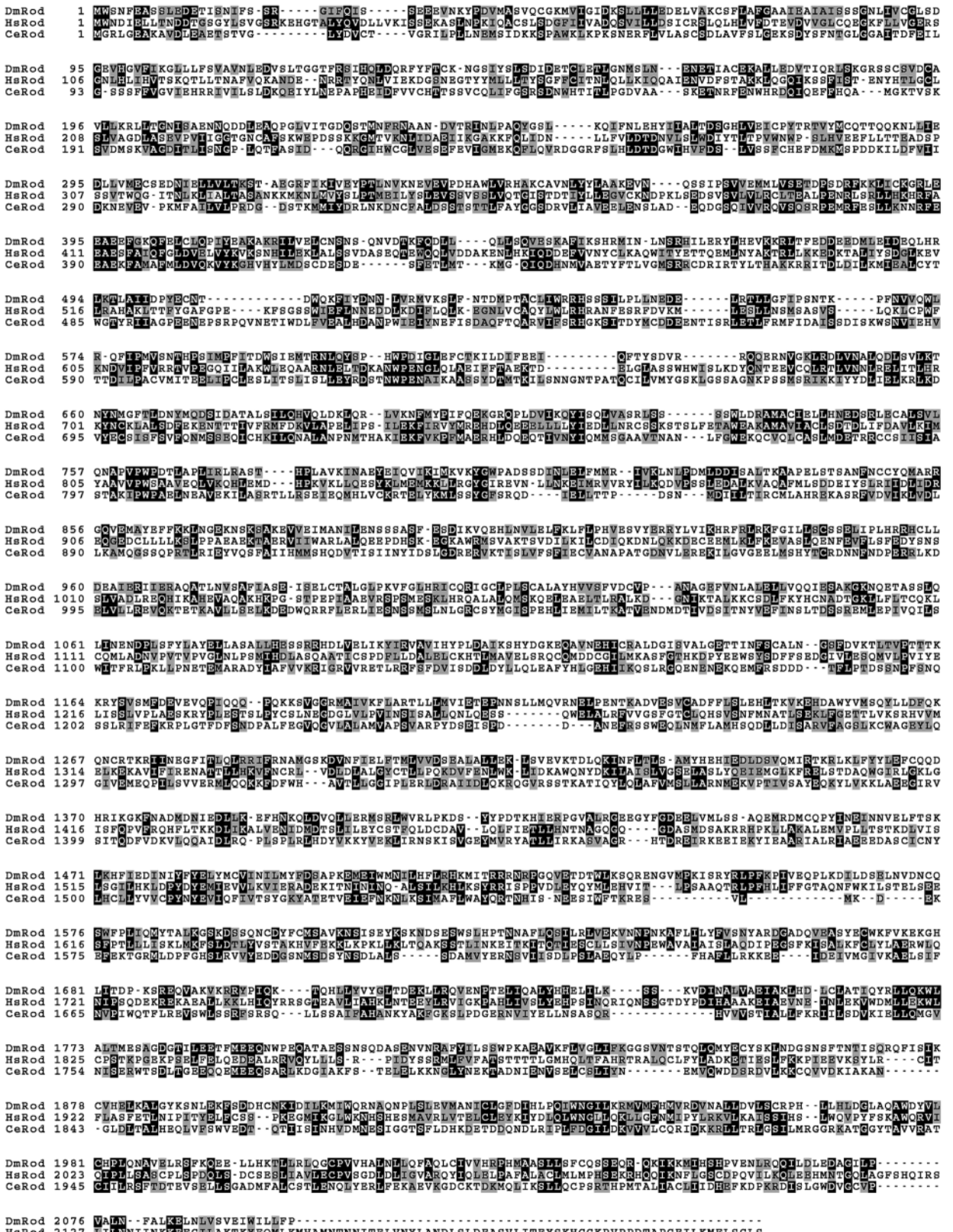

Fig. 3. Homologs to Drosophila *rough deal* are found in the genomes of humans and worms. Multiple sequence alignment of a portion of Rod proteins from *Drosophila* (DmRod), *H. sapiens* (HsRod) and *C. elegans* (CeRod). Identical amino acids between any two genes are shaded black, conservative substitutions are shaded gray. This alignment used CLUSTAL W. (Thompson et al., 1994). GenBank accession numbers for Hsrod and Cerod are P50748 (Nagase et al., 1996) and AAB00646 (Wilson et al., 1994), respectively.

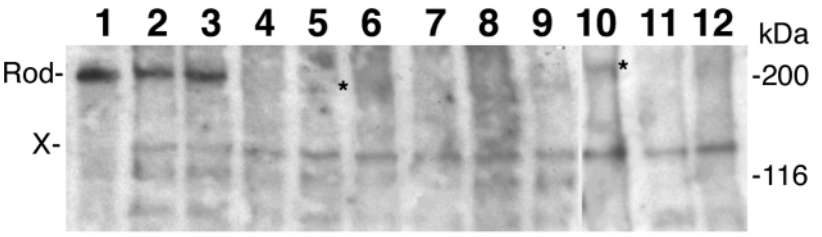

Fig. 4. Detection of the Rod protein by immunoblot. The serum recognizes a large ca. 200 kDa protein in protein extracts of *Drosophila* Schneider Line 2 tissue culture cells (lane 1), as well as in wild-type larval brains (lane 2). In the transgenic fly stock *C3L9*, containing 2 additional copies of the wild-type *rod* gene, the 200 kDa Rod protein is visibly more abundant than in wild-type brains (compare with lane 2), while the other bands in the lane are unchanged (lane 3). Rod protein is not detectable in *rod* alleles *X1, H4.8, 29D, RP153, X2, X4* and *X6* (lanes 4, 6-9, 11, 12). In these lanes, only the 200 kDa protein is specifically eliminated by the *rod* mutation. In two alleles, *X5* (lane 5) and *X3* (lane 10), Rod protein is detectable (indicated by *), though in reduced abundance. The protein in rod^{X5} is slightly smaller than wild type, as predicted from its structure. The band labeled X is present in all extracts except the S2 cells and is apparently unrelated to Rod, as its abundance is unaltered in the various rod mutants and the transgenic line. It serves here as a loading control.

alleles examined lacked this 200 kDa band. The rod^{X3} allele, however, consistently displayed a reduced amount of the 200 kDa protein. In the alleles lacking the 200 kDa Rod protein, no new allele-specific variants were consistently recognized by the antiserum, with the exception of rod^{X5}, which presented a very weak band migrating slightly faster than the wild-type band. This allele with its in-frame deletions totaling 201 bp would be expected to produce a protein 7.5 kDa smaller than wild-type Rod.

Thus all the mutant alleles examined with the exception of rod^{X3} and rod^{X5} appear to be protein nulls. Moreover, immunostaining (see below) of rod^{X3} and rod^{X5} cells detected no concentration of Rod protein associated with the mitotic apparatus. These results are consistent with the fact that all these alleles behave as genetic nulls: no difference in phenotype was seen in individuals with two doses of a given mutant allele when compared to those with one dose in combination with the rod^{X1} deletion (data not shown).

Distribution of Rod in meiotic spermatocytes

We examined by immunocytochemistry wild-type meiotic spermatocytes that had been stained with the anti-Rod antiserum in order to observe the intracellular distribution of Rod protein during cell division. Primary spermatocytes are among the largest dividing cells of the *Drosophila* life cycle, and the first meiotic division provides particularly favorable cytology for observing chromosome behavior and spindle structure. The spindles are also simpler than mitotic spindles since the chromosome pairs form 4 bivalent elements, with a total of only 4 pairs of kinetochores. In addition, these cells also have an extended prophase and prometaphase period compared to mitotic tissues (Cenci et al., 1994).

In interphase and prophase of the first meiotic division, there was no obvious concentration of Rod protein. At prometaphase, the 4 bivalents are seen as 3 large DAPI-staining discrete blobs (the *XY* pair and the pairs of the large chromosomes *2* and *3*) plus a small dot (the chromosome *4* pair). At this stage, before the chromosomes have completed their attachment to the spindle, Rod was detected on two bright, well-defined spots of each bivalent mass (Fig. 5A,E). These are most likely the kinetochores, since similar staining is seen with known *Drosophila* kinetochore components such as Zw10 (Williams et al., 1996) and cytoplasmic dynein (Starr et al., 1998, and data not shown). In some cases spindle fibers could be seen terminating in these bright spots at prometaphase (Fig. 5E).

By metaphase, the bivalents are bioriented and aligned on the meiosis I metaphase plate (Fig. 5B,F). In such cells Rod protein was no longer exclusively at the kinetochore, but instead was found in an uneven, fibrous or bead-like distribution along the K-MT bundles. Once anaphase began, Rod was gone from the K-MT bundles and was found exclusively on the kinetochores as the chromosomes moved to the poles (Fig. 5C,G). Rod remained at the kinetochores throughout anaphase, but slowly disappeared towards the end of telophase I (Fig. 5H).

The behavior of Rod in the second meiotic division resembled that seen in the first division. It was found in pairs of dots around the centromeres in prometaphase II (Fig. 5I). In metaphase Rod was found distributed along the K-MT fibers of the spindle (Fig. 5J). Immediately after the onset of anaphase (Fig. 5K,L), Rod was once again restricted to the kinetochore region of the migrating chromatids, and as the cell completed telophase the signal faded away (Fig. 5M). In cells of homozygous *rod* mutant individuals, no specific staining was seen either of kinetochores or spindle structures at any stage of meiosis (an early anaphase of rod^{X3} is shown in Fig. 5N).

Rod localization in mitotic neuroblasts

We also examined by immunofluorescence the intracellular localization of Rod in squashed preparations of mitotically dividing neuroblasts of wild-type third instar larval brains (Fig. 6). Here, the first discrete labeling of Rod was visible only at metaphase (Fig. 6A,B), where the irregular distribution of the protein along K-MT bundles was particularly evident. At anaphase (Fig. 6C-E), this K-MT labeling disappeared, and Rod was found exclusively in the region of the kinetochores at the leading edges of the migrating chromatids, where it remained until the end of telophase, as described above for the spermatocyte meiotic division. Fig. 6E shows a particularly early anaphase in which a small amount Rod material seemed to be still associated with the K-MTs.

Because it is normally difficult to identify mitotic neuroblasts in prometaphase (i.e. before all the chromosomes have become bioriented at the spindle equator), we examined colchicine-treated neuroblasts, since such treatment leads to a rapid arrest of mitotic cells in a prometaphase like state (Gonzalez et al., 1991). The chromosomes of colchicine-treated cells stained brightly with the anti-Rod serum usually in the double-dot pattern characteristic of kinetochore proteins (Fig. 6F). Although it is difficult to be quantitative in judging

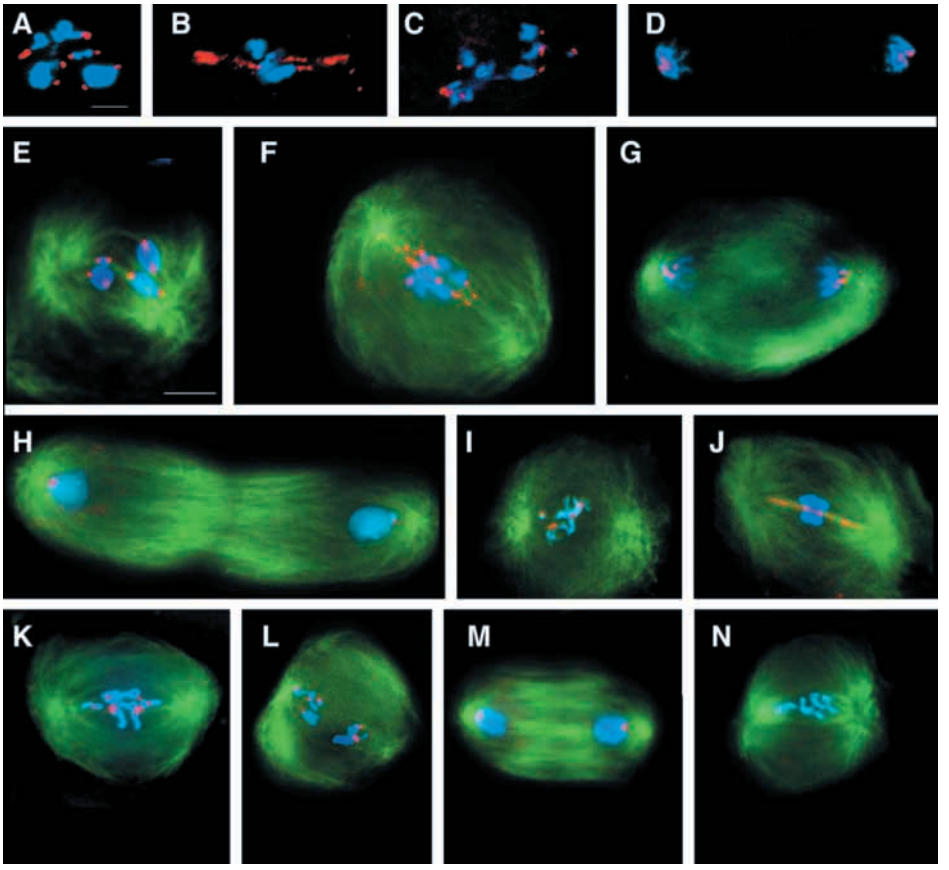

Fig. 5. Immunolocalization of the Rod protein during the first and second meiotic divisions in wild-type spermatocytes. (A-D) Double labeling for Rod protein (red) and DNA (blue) during the first meiotic division. (A) prometaphase; (B) metaphase; (C) early anaphase; (D) late anaphase/early telophase. (E-H) Triple labeling for Rod (red), DNA (blue), and tubulin (green) during meiosis I. (I-N) Triple labeling of meiosis II cells. Rod protein localizes to kinetochores at prometaphase. A pair of Rod-containing dots is found on each of the four bivalent masses in the prometaphase figures of A and E. In E, some kinetochores appear to have already captured microtubules. The kinetochores of prometaphase II chromosomes (I) are also stained by anti-Rod. At metaphase (B,F,J) Rod distributes irregularly along K-MT fibers. Colocalization of the green microtubules and the red Rod protein is shown in yellow. At early anaphase (C,K) Rod is found exclusively at the leading edge of the migrating chromosomes (C) and chromatids (K), and remains associated as anaphase progresses (D,G,L). By telophase (H,M), Rod labeling of kinetochores begins to disappear. No specific labeling is found in cells of *rod* mutants such as rod^{X3} (N), shown here in anaphase II. Bars: (A) 5 μm, for A-D; (E) 5 μm, for E-M.

the intensity of the staining, Rod protein accumulation in the kinetochore regions of these condensed chromosomes often appeared to be greater than the Rod protein associated with untreated chromosomes.

In summary, the same dynamic changes in Rod distribution are seen in both meiosis and in mitosis: Rod is localized specifically to kinetochores in the prometaphase-like state of colchicine treated cells, it is found on kinetochore fibers at metaphase, but it redistributes back to kinetochores at anaphase. Rod is also found specifically on the kinetochores of the prometaphase-like state of colchicine treated cells. The association with K-MTs seems to be specific, since other MTs in the spindle do not contain detectable amounts of Rod. Significantly, in all of these details, the behavior of Rod exactly parallels the described behavior of the Zw10 protein (Williams et al., 1992, 1996; Williams and Goldberg, 1994).

Colchicine and taxol treated *rough deal* cells undergo premature sister chromatid separation

Drosophila neuroblasts from *zw10* mutant individuals show a greatly elevated frequency of premature sister chromatid separation (PSCS) reaching some 30%, when treated with the colchicine or taxol (Williams et al., 1992). Such a phenotype was not reported in the original description of *rod* mutants (Karess and Glover, 1989), but because the phenotypes of *rod* and *zw10* and the behavior of their corresponding proteins are similar in other respects, we reexamined the behavior of *rod* cells in the presence of these microtubule poisons.

We found that a 30 minute treatment of *rod* mutant brains with either colchicine or taxol at 10 μM, followed by a 5 minute hypotonic shock, induces PSCS in a significant fraction of the mitotic cells (20-30%). This PSCS often involved all the chromosomes in a given cell, but cells where only some chromatids had separated were also frequently seen (Fig. 7). Identical treatment of wild-type brains only rarely produced PSCS (<1%). Thus, the similarity of the *rod* and *zw10* mutant phenotypes extends to their response to these microtubule poisons.

DISCUSSION

The *rough deal* protein is a kinetochore component

We have shown in this study that the *rough deal* gene encodes a new, evolutionarily conserved 240 kDa kinetochore and K-MT associated protein. The mitotic phenotype of *rod* mutants (Karess and Glover, 1989) when considered together with the changes in subcellular distribution of Rod protein during mitosis described here, argues that Rod acts at the kinetochores and kinetochore microtubules to provide an activity required for the coordinate separation or movement of sister chromatids at anaphase onset.

Rod now joins Zw10 on the list of proteins whose association with metazoan kinetochores changes as mitosis progresses. Among these are Mad2 (Chen et al., 1996; Li and Benezra, 1996), Cdc20 (Kallio et al., 1998), Bub1 and Bub3 (Basu et al., 1998; Taylor et al., 1998) implicated in the spindle assembly checkpoint, microtubule motor proteins such as the kinesin CENP-E (Cooke et al., 1997; Yao et al., 1997), and the cytoplasmic dynein-dynactin complex (Echeverri et al., 1996;

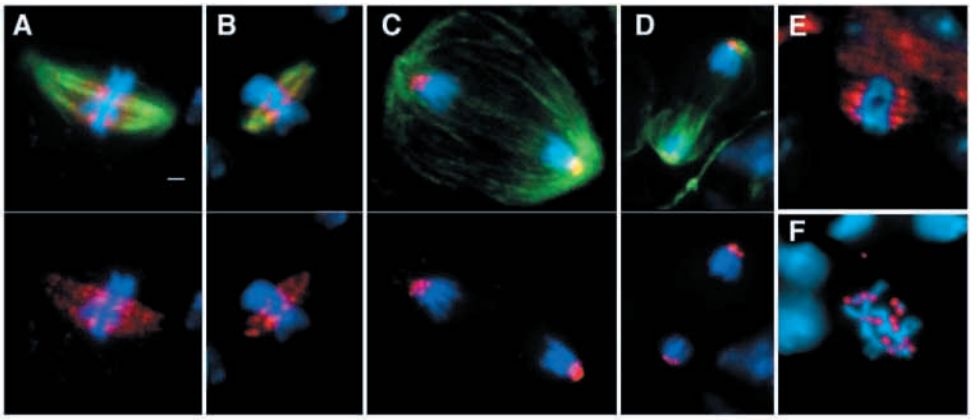

Fig. 6. Immunolocalization of the Rod protein during mitosis in wild-type larval neuroblasts. In A-D, the top panels are triple labeled for Rod (red), DNA (blue) and tubulin (green). The bottom panels show the same cells without the tubulin, to better reveal the bead-like distribution of Rod. In metaphase (A,B), Rod labels the K-MTs over nearly their entire length. By anaphase (C-E), Rod rapidly relocalizes to the kinetochores, and disappears during telophase. (E) A very early anaphase in which a small amount of Rod material still lingers on the K-MTs. (F) Colchicine-treated cells are arrested in a prometaphase like state, and 8 pairs of Rod-containing dots can be seen associated with the chromosomes, presumably at the kinetochores. Bar, 1 μm.

Pfarr et al., 1990; Starr et al., 1998; Steuer et al., 1990), and the microtubule binding protein CLIP 170 (Dujardin et al., 1998).

Rod is a conserved protein

Although the *Drosophila* Rod sequence offers no hint of its function, having no identifiable peptide motifs associated with known activities in other proteins, it does have clear homology to genes in nematodes and humans, two species widely separated by evolution. This protein is likely therefore to be an important kinetochore component in all metazoans. The Zw10 protein too is conserved across phyla. In fact, the Zw10 homologs of worm and human have been shown to retain at least some of the activity expected of them if they are indeed performing functions similar to those seen in the fly (Starr et al., 1997). We have preliminary evidence that this may be true as well for the Rod homologs (F. Scaërou and R. Karess, unpublished).

Neither *rough deal* nor *zw10* appears to have related genes in budding yeast. If this absence has functional significance, that is, if mitosis in yeast is different in some way that obviates the need for the functions provided by Rod and Zw10, perhaps it is related to the fact that there seems to be no stage in yeast mitosis corresponding to the alignment of chromosomes on the metaphase plate (Straight et al., 1997).

The dynamics of Rough Deal distribution

The changes in Rod protein localization are best illustrated in the spermatocyte first meiotic division, although the essential features of Rod dynamics are also found in meiosis II and in mitotic neuroblasts of third instar larvae and are therefore likely to be common to all dividing cells. Rod is seen to associate first with kinetochore regions of prometaphase bivalents, but once chromosomes are aligned on the metaphase plate, the protein is found irregularly distributed along much of the length of the K-MTs. There is no detectable Rod associated with spindle structures prior to this moment. Although it is difficult to say with certainty whether a given bivalent is entirely unattached to the spindle or is mono-oriented, we never saw unilateral Rod labeling of a K-MT bundle. That is, either both K-MT bundles emanating from the two kinetochores of a given bivalent were labeled, or none were. This suggests that Rod does not change its location simply in response to MT capture by the kinetochore, but that biorientation is required.

Concurrently with the onset of anaphase, Rod rapidly disappears from the K-MTs and is seen once again exclusively on the kinetochores of the now poleward-migrating chromosomes. The transition from the metaphase staining of K-MTs to the anaphase staining of kinetochores must occur very rapidly, because we have only rarely seen intermediate forms of Rod distribution such as that shown in Fig. 6E, that is, an anaphase cell in which some K-MT staining persists. Rod remains associated with kinetochores as anaphase progresses, and it slowly disappears at the end of telophase. There does not appear to be any subsequent association of Rod with the remaining spindle structures, such as the midbody or the poles.

Although these observations suggest that the Rod protein accumulates at the kinetochore, moves along the K-MT once the chromosomes are bioriented, and then returns to the kinetochore at anaphase onset, there is no direct evidence that a given Rod molecule migrates from the kinetochore to the K-

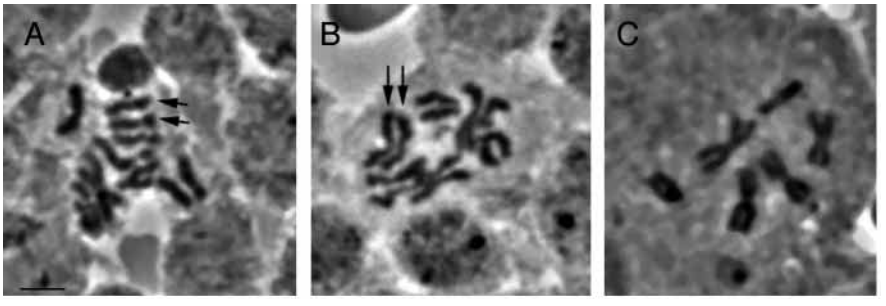

Fig. 7. Premature sister chromatid separation (PSCS) in *rod* mutant cells treated with microtubule poisons. Approximately 20-30% of all metaphases in such cells display PSCS. (A,C) Colchicine treatment; (B) Taxol treatment. (A,B) rod^{X2} cells, (C) wild type. Arrows indicate separated sister chromatids. Note that not all the chromosomes in a given cell are affected. Bar, 2 μm.

MT and back again. It is possible for example that during metaphase there is a unidirectional flux of Rod-containing material which continually accumulates at the kinetochore, but then periodically sheds off and travels along the K-MTs towards the pole, eventually falling off the spindle. Such a behavior might account for the lumpy distribution of Rod along the K-MTs in metaphase cells (particularly evident in Fig. 5). It might also explain the variability observed in the intensity of anti-Rod staining material at the K-MT/chromosome junction in metaphase, which sometimes is the most prominent spot along each kinetochore fiber but which is relatively poorly marked at other times (Fig. 6A,B, and data not shown). Observing a fluorescently-tagged Rod protein in vivo should help us to understand these dynamics in more detail.

Rough deal may function together with Zw10

Given that *rod* and *zw10* null mutants have similar mitotic phenotypes (lagging chromatids at anaphase of untreated cells (Karess and Glover, 1989; Williams et al., 1992), and premature sister chromatid separation in cells treated with taxol or colchicine (Williams et al., 1992; this report)), it would not be surprising if the proteins encoded by these two genes acted together in the same pathway to carry out their functions. There is growing evidence that this is indeed the case. Firstly, our description of Rod protein behavior during mitosis and meiosis is identical to that reported for Zw10 (Williams et al., 1992, 1996; Williams and Goldberg, 1994). In addition (Williams and Goldberg, 1994) reported that in a *rod* mutant background, Zw10 protein no longer localizes on kinetochores or K-MTs. We can also add a number of recent unpublished observations supporting this hypothesis: (i) Rod no longer localizes to kinetochores in a *zw10* mutant background; (ii) a double *rod*; *zw10* mutant has no worse phenotype than either mutant alone; (iii) there is evidence of a physical complex containing both proteins (F. Scaërou, D. Starr, R. Karess and M. Goldberg, unpublished).

Starr et al. (1998) have found that *rod* and *zw10* activity are both required to recruit cytoplasmic dynein to the kinetochore. The Zw10 protein at least appears to be directly involved, since it is capable of interacting with the p50 subunit of the dynein-dynactin complex in the yeast 2 hybrid system (Starr et al., 1998). Since Rod and Zw10 proteins behave so similarly, and their mutant phenotypes are indistinguishable, it is likely that they are both part of this recruitment complex. Some, and perhaps all of the phenotypes associated with *rod* and *zw10* mutants may then be attributable to the absence of cytoplasmic dynein from the kinetochore. This, as discussed by Starr et al. (1998) may call for a reassessment of the role of cytoplasmic dynein at the kinetochore, since it has heretofore been considered important primarily in prometaphase events such as chromosome capture and congression, which appear to function normally in *rod* and *zw10* mutants. We cannot exclude, however, that Zw10 and Rod have additional functions, or that their functions may not be completely identical.

The described *rod* mutant phenotype is the null phenotype

As far as can be determined by western blotting, at least 7 of the 9 mutant alleles examined here are protein nulls, and none of the 9 alleles showed detectable immunostaining of kinetochores or K-MTs. The antibody employed in this study was raised against the N-terminal 40 kDa of Rod, and should in principle have detected any stable Rod-related protein encoded by the alleles mutated downstream of this region, as is the case for rod^{RP153}, $rod^{H4.8}$, rod^{29D} and rod^{X5}. While it is easy to imagine the transposon insertions of rod^{RP153}, $rod^{H4.8}$, rod^{29D} interfering with transcription or processing of the message, as well as potentially truncating the protein, the known lesions in rod^{X5} remove or alter only 69 amino acid residues, but leave the reading frame intact. There may be a very small amount of a Rod-related protein in this mutant (Fig. 4), but at only barely detectable levels. This may indicate that the altered and missing sequences of rod^{X5} are critical to Rod stability. We caution, however, that only the 500 bases immediately around the deleted region in this allele were sequenced, and the gene might have sustained other changes elsewhere. A second allele, rod^{X3} expresses low but easily detectable amounts of protein, which appears incapable of localizing to the kinetochores. The lesion in this allele has not been mapped, but its position ought to identify a functionally important domain of the Rod protein.

The previous ranking of the extent alleles (Karess and Glover, 1989) held rod^{X1} to be the most severe since its lethal phase was early pupal rather than late pupal. We now know from the molecular structure that the deletion in this particular allele, in addition to removing the entire amino-terminal half of *rod*, also removes a portion of the intergenic (presumably regulatory) region between *rod* and the adjacent transcription unit. The 100% penetrant early pupal lethality may be a consequence of simultaneously eliminating *rod* and reducing the expression of its neighbor.

Knowing that the *rod* phenotype we have described is the null phenotype is important for understanding its role in mitosis. The majority of chromosomes in *rod* mutant cells still disjoin normally. In other words, the function of Rod must be one that augments the fidelity or coordination of sister chromatid segregation, but is not critical to this process. Although Rod protein is present on kinetochores during prometaphase and K-MTs during metaphase, it would seem to play no essential role in pre-anaphase events such as the initial attachment of chromosomes to the spindle or in their congression to the equator since in *rod* mutants these events appear to be unperturbed. Perhaps Rod carries out some function during this period the consequences of which are only evident at the onset of anaphase. For example it may be involved in the assembly of some aspect of kinetochore structure which is only utilized during anaphase. Alternatively, Rod may function at both metaphase and anaphase, but its metaphase-specific functions might be entirely redundant with those provided by other gene products, thus yielding no *rod*-associated phenotype, while the anaphase functions might be only partially so, and therefore are manifest in *rod* mutants.

What is the function of Rod and Zw10 in mitosis?

Williams et al. (1996) have demonstrated that the global behavior of Zw10 as the cell progresses from prometaphase to metaphase to anaphase appears to be a response to changes in tension exerted by the spindle across the kinetochores. Such tension is absent from the kinetochores of unattached or mono-oriented chromosomes at prometaphase, of migrating chromatids or chromosomes at mitotic and meiotic anaphase,

and of chromosomes in cells treated with colchicine, and in each of these situations Zw10 is found only on kinetochores. Only properly bioriented chromosomes or bivalents aligned on the metaphase plate are subject to bipolar tension across the kinetochores, and Zw10 is found only on the K-MTs that appear to be attached to these chromosomes. The Rod protein behaves identically to Zw10 in this regard. Thus both proteins may be part of a mechanism that senses this correct biorientation, either by responding to bipolar tension (Li and Nicklas, 1995), or perhaps by monitoring microtubule occupancy (Waters et al., 1998).

It is difficult to explain simultaneously the lagging chromatid phenotype of *rod* and *zw10* mutants and the PSCS phenotype seen in colchicine- and taxol-treated mutant cells. These phenotypes appear at first glance contradictory: the absence of Rod or Zw10 appears to retard chromatid separation in untreated cells, but promotes their premature separation in the presence of the drugs. How this should happen, and what this tells us about wild-type *rod* and *zw10* function are not clear. Although colchicine depolymerizes microtubules and taxol stabilizes them, both treatments have the net effect of reducing tension across the kinetochore (Waters et al., 1998) which delays anaphase onset by maintaining the activity of the checkpoint. Taken alone, the PSCS phenotype suggests that SAC is slightly defective in *rod* and zw10 mutants, or can be more easily circumvented, since a failure to arrest in metaphase in response to spindle damage by drugs is a hallmark of mutations affecting components of the SAC. This would imply that Rod and Zw10 are ancillary components of the checkpoint mechanism, or perhaps that *rod* and *zw10* mutant chromosomes are hypersensitive to activity of the anaphase promoting complex (APC), which is normally suppressed by the SAC.

One way to think about Rod's function would be as a part of a process that prevents premature entry into anaphase. In untreated cells, the absence of Rod (or Zw10) would allow anaphase to start before certain chromosomes were 'ready' in some as yet undefined way. (Readiness might for example mean reducing but not eliminating the cohesiveness of sister chromatids in order to render them more sensitive to APC activity). As a result, the chromatid sisters of unprepared chromosomes would separate later than those of chromosomes that were 'ready'. In wild-type drug-treated cells, the chromosomes might have time to become 'ready' to separate in the above sense, but are arrested by the activity of the SAC. In the absence of the monitoring by Rod and Zw10 however, the cell may prematurely activate the APC, overriding the delay imposed by the SAC. This model is vague, but it has the virtue of postulating a single function for the Rod/Zw10 complex, that of preventing premature entry into anaphase. Given that Rod and Zw10 do seem to be at least useful for maintaining the arrest in metaphase of colchicine-treated cells, they can be considered in a formal sense as part of a checkpoint mechanism, as has been noted by Williams and Goldberg (1994). However, it would have to be one of only secondary importance, since chromosomes apparently do align correctly on the metaphase plate in *rod* and *zw10* mutants, and there does not appear to be any obvious change in the timing of mitotic events.

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