Megator, an Essential Coiled-Coil Protein that Localizes to the Putative Spindle Matrix during Mitosis in Drosophila

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We have used immunocytochemistry and cross-immunoprecipitation analysis to demonstrate that Megator (Bx34 antigen), a Tpr ortholog in Drosophila with an extended coiled-coil domain, colocalizes with the putative spindle matrix proteins Skeletor and Chromator during mitosis. Analysis of P-element mutations in the Megator locus showed that Megator is an essential protein. During interphase Megator is localized to the nuclear rim and occupies the intranuclear space surrounding the chromosomes. However, during mitosis Megator reorganizes and aligns together with Skeletor and Chromator into a fusiform spindle structure. The Megator metaphase spindle persists in the absence of microtubule spindles, strongly implying that the existence of the Megator-defined spindle does not require polymerized microtubules. Deletion construct analysis in S2 cells indicates that the COOH-terminal part of Megator without the coiled-coil region was sufficient for both nuclear as well as spindle localization. In contrast, the NH2-terminal coiled-coil region remains in the cytoplasm; however, we show that it is capable of assembling into spherical structures. On the basis of these findings we propose that the COOH-terminal domain of Megator functions as a targeting and localization domain, whereas the NH2-terminal domain is responsible for forming polymers that may serve as a structural basis for the putative spindle matrix complex.

INTRODUCTION

Although much work has been directed toward understanding mitotic spindle apparatus structure and function, it is still unclear how mechanical forces are applied to pull the chromosomes to the spindle poles (Pickett-Heaps et al., 1982; 1997; Scholey et al., 2001). The involvement of a spindle matrix that can act as a stationary substrate to stabilize the spindle during force production, and microtubule sliding has long been proposed (Pickett-Heaps et al., 1982; 1997); however, direct evidence for its existence has remained elusive (Scholey et al., 2001; Wells, 2001; Bloom, 2002; Kapoor and Compton, 2002; Johansen and Johansen, 2002). Recently, a putative spindle matrix protein, Skeletor, was identified in Drosophila (Walker et al., 2000). Skeletor is associated with chromosomes at interphase, but preceding microtubule spindle formation and nuclear lamina breakdown, it redistributes into a true fusiform spindle at prophase. During metaphase the Skeletor defined spindle and the microtubule spindles are coaligned and when embryos are treated with nocodazole to disassemble microtubules, the Skeletor spindle persists (Walker et al., 2000). Thus, many of the features of the Skeletor defined spindle are consistent with the spindle matrix hypothesis. Using a yeast two-hybrid screen with Skeletor sequence as bait Rath et al. (2004) identified another potential component of a spindle matrix, Chromator, that interacts directly with Skeletor. Chromator contains a chromodomain and colocalizes with Skeletor on the chromosomes at interphase as well as to the Skeletor-defined spindle during metaphase. Furthermore, functional assays using P-element insertion mutants and RNAi in S2 cells suggest that Chromator is an essential protein that affects spindle function and chromosome segregation (Rath et al., 2004).

The above findings support the hypothesis that Skeletor and Chromator are members of a macromolecular spindle matrix complex constituted by several nuclear components (Walker et al., 2000; Rath et al., 2004). However, for a spindle matrix to form independently or to form a structural scaffold aligned with the microtubule spindle, one or more of its molecular components would be predicted to have the ability to form polymers. Neither Skeletor nor Chromator appears to contain molecular motifs with such properties. In this study we report the identification of another molecular component that localizes to the putative spindle matrix and is a candidate to play such a structural role. The mAb Bx34 was previously shown to recognize a 260-kDa protein with a large NH2-terminal coiled-coil domain and a shorter COOH-terminal acidic region that shows overall structural and sequence similarity to the mammalian nuclear pore complex Tpr protein (Zimowska et al., 1997). Zimowska et al. (1997) showed that the Bx34 antigen during interphase was localized to the nuclear rim as well as occupying the intranuclear space surrounding the chromosomes. Here we show using immunocytochemistry and analysis of P-element mutations that the Bx34 antigen is an essential protein that colocalizes with Skeletor and Chromator to the putative spindle matrix as it is defined by these proteins during
Mitosis. Furthermore, based on the presence of the large coiled-coil domain, we propose the Bx34 antigen may serve as a structural component of the spindle matrix and have named the protein Megator.

**MATERIALS AND METHODS**

**Drosophila Stocks**

Flies were maintained according to standard protocols (Roberts, 1986). Oregan-R or Canton-S was used for wild-type preparations. The y w 1; P[w+; lacW2(3R)009505/Oy] line was obtained from the Bloomington Stock Center and was originally part of the István Kiss collection (Trk et al., 1993). To facilitate identification of homozygous mutant Megator embryos, P[w+; lacW2(3R)009505/Oy] was balanced over one of two different green fluorescent protein (GFP) lines, CyO; Cy3B/Y or CyO; Cy2B/3B, obtained from the Bloomington Stock Center line: w*; Int[2R]NtocScore9Rb, Int[Cy2]3B, P[w+; lac-W2(3R)009505/Oy].

**Antibodies**

Residues 1433–1703 of the predicted Megator protein were subcloned using standard techniques (Sambrook et al., 1989) into the pGEX-4T-1 vector (American Type Culture Collection, Rockville, MD). The mouse IgG1 on-column purified from mouse IgG1 or IgM (Sigma-Aldrich, St. Louis, MO), according to the pGEX manufacturer’s instructions (Amersham Pharmacia Biotech). The mAbs 12F10 and 11E10 were generated by injection of 50 μl of 3–4 h embryonic lysate on a rotating wheel. Beads were washed three times for 10 min each with 1 ml of ip buffer with low-speed pelleting of beads between washes. The resulting bead-bound antibodies were analyzed by SDS-PAGE and Western blotting according to standard techniques (Harlow and Lane, 1988) using mAb 6H11 to detect Chromo- mator and mAb 12F10 to detect Megator.

**Immunohistochemistry**

Antibody labelings of 0–3 h embryos were performed as previously described (Johansen et al., 1996; Johansen and Johansen, 2003). The embryos were dechorionated in a 50% Chlorox solution, washed with 0.7 M NaCl/0.2% Triton X-100 and fixed in a 1:1 heptane:fixative mixture for 20 min with vigorous shaking at room temperature. The fixative was either 4% paraformaldehyde in phosphate-buffered saline (PBS) or Bouin’s fluid (0.66% picric acid, 9.5% formalin, 4.7% acetic acid). Vitelline membranes were then removed by shaking embryos in heptane-methanol (Mischton and Sedat, 1983) at room temperature for 30 s. S2 cells were fixed onto poly-L-lysine–coated coverslips and fixed with Bouin’s fluid for 10 min at 24°C and methanol for 5 min at −20°C. The cells on the coverslips were permeabilized with PBS containing 0.5% Triton X-100 and incubated with diluted primary antibody in PBS containing 0.1% Triton X-100, 0.1% sodium azide, and 1% normal goat serum for 1.5 h. Double and triple labelings employing epifluorescence were performed using various combinations of antibodies against Megator (mAb 12F10, IgG1), Chromator (mAb 6H11, IgG1), Skeletor (mAb 1A1, IgM), anti-tubulin antibody (mouse IgG1 on-column purified from mouse IgG1 or IgM, IgG1), and Hoescht to visualize the DNA. The appropriate species and isotype specific Texas Red-, TRITC-, and FITC-conjugated secondary antibodies (Cappel/ICN, Standards Biotechnology, Bir- mmington, MN) were used to visualize primary antibody labeling. Confocal microscopy was performed with a Leica confocal TCS NT microscope system (Deerfield, IL) equipped with separate argon-UV, argon, and krypton lasers and the appropriate filter sets for Hoechst, FITC, Texas Red, and TRITC imaging. A separate series of confocal images for each fluorophor of double-labeled preparations were obtained simultaneously with z-intervals of typically 0.5 μm using a PL APO 100×/1.40–0.70 oil objective. A maximum projection image for each of the image stacks was obtained using the Imaging software. The fixation was either 4% paraf- formaldehyde or 2% paraformaldehyde in phosphate-buffered saline (PBS) or Bouin’s fluid for 10 min at 24°C. Images were imported into Photoshop where they were pseudocolored, image processed, and merged (Adobe Systems, San Jose, CA). In some images nonlinear ad- justments were made for optimal visualization especially of Hoescht labelings of nuclei and chromosomes. Polyethylene chromosome squash preparations from late third instar larvae were immunostained with the Skeletor antibody mAb 1A1 and Megator antibody mAb 12F10, essentially as previously described by Zink and Paro (1999), Jin et al. (1999), and Wang et al. (2001).

**Microtubule Depolymerization Experiments**

Dechorionated embryos from 0–2.5 h collections were added to heptane containing 10 μM nocodazole (Sigma-Aldrich) and shaken for 1.5 min, before adding fixative and incubating for an additional 20 min. Cold-treated em- bryos were incubated at 0°C for 30 min and incubated for 1 min with prechilled heptane. Prechilled Boulin’s fluid was then added to the heptane layer, shaken for 30 s, and rotated for 4°C for 20 min. Immunolabeling was performed as described above.

**Expression of Megator Constructs in Transfected S2 Cells**

A full-length Megator (2346 aa) construct, a N-terminal domain construct of Megator from residue 1–1431 containing 87% of the coiled-coil region, and a COOH-terminal domain construct of Megator from residue 1758–2346 were cloned into the pMT/V5-HisB vector (Invitrogen) with in-frame V5 tags at the COOH-termini using standard methods (Sambrook et al., 1989). Similarly, a middle construct from residue 1432–1709 was subcloned into the pMT/V5- HisA vector with an in-frame V5 tag at the COOH-terminus. The reducibility of all constructs was verified by sequencing at the Iowa State University Sequencing facility.

**Drosophila Schneider 2 (S2) cells were cultured in**

Shields and Sang M3 insect medium (Sigma) supplemented with 10% fetal or newborn bovine serum, antibiotico/antimycotic solution, and l-glutamine (Life Technologies, BRL/Life Technologies) at 25°C. The S2 cells were transfected with different Megator subclones using a calcium phosphate transfection kit (Invitrogen), and expression was induced by 0.5 mM CuSO4. Cells expressing Megator constructs were harvested 12–24 h after induction and washed onto poly-L-lysine–coated coverslips for immunostaining and Hoescht labeling.

**RNAi Interference**

dsRNA in S2 cells was performed according to Clemens et al. (2000). A 784-base pair fragment encoding sequence from the coiled-coil region of Megator DNA was PCR amplified on 100 μl of S2 cells in 6–12 h. dsRNA was transcribed using the Megascript RNAi kit (Ambion, Austin, TX). Synthesized dsRNA, 40 μg, was added to 1 × 107 cells in six-well cell culture plates.
Control dsRNAi experiments were performed identically except pBluescript vector sequence (800 base pairs) was used as template. The dsRNA-treated S2 cells were incubated for 120 h and then processed for immunostaining and immunoblotting. For immunoblotting 10^5 cells were harvested, resuspended in 50 μl of S2 cell lysis buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, and 1% Nonidet P-40), boiled, and analyzed by SDS-PAGE and Western blotting with anti-Megator antibody (mAb 12F10) and anti-α tubulin antibody. The mitotic index defined as the number of cells in metaphase and anaphase as a percentage of total cell number were compared between experimental and control S2 cell cultures. At least 500 cells were examined in each individual experiment (range: 500-2500 cells).

Analysis of P-element Mutants

PCR Mapping. The insertion site flanking sequence provided by the Berkeley Drosophila Genome Project for the P[w^+]mc = lacW/2Rk3905/ACR03 element (Accession no. AQQ025733) placed the P-element insertion near the transcription start site for the Megator gene. By designing several sets of nested forward and reverse primers from genomic sequence encompassing this region, we performed PCR from mutant flies as previously described (Preston and Engels, 1996). PCR fragments were subcloned and sequenced according to standard protocols (Sambrook et al., 1989).

Viability Assays. To determine the viability of Megator mutants, we analyzed the offspring from crosses of l(2)k03905/CyO, P[w^+]mc = Act-GFP]JMR1 parents in which the balancer chromosome is labeled with GFP, allowing for the identification of homozygous l(2)k03905/l(2)k03905 embryos and larvae. For these assays eggs were collected on standard yeasted agar plates and incubated at 21°C. No homozygous l(2)k03905/l(2)k03905 larvae were found among 200 third instar larvae examined from such crosses, and among 300 embryos only one homozygous l(2)k03905/l(2)k03905 first instar larvae emerged.

P-element Excision. The P element of y1 w67c23; P[w^+]mc = lacW/2Rk3905/ACR03 was mobilized by a Δ2–3 transposase source (y^A w^A; CyO, Ht[w^+]mc = PΔ2–3/Hod2.1/BeC/Egr^P2; Robertson et al., 1988). Several fly lines in which the P element had been excised were identified by their white eye color. Three precise excisions were confirmed by PCR analysis using primers corresponding to the P element and/or the genomic sequences flanking it. DNA isolation from single flies and PCR reactions were performed as
described in Preston and Engels (1996). The precise excision lines were further analyzed for viability as described above and for restoration of Megator protein levels by immunoblotting. Protein extracts were prepared by homogenizing adult flies in IP buffer. Homozygous excised \( l(2)k03905 \) flies were identified by the absence of the Curly marker. Proteins were separated on SDS-PAGE and analyzed by Western blotting with anti-Megator antibody (mAb 12F10) and anti-\( \alpha \)-tubulin antibody.

RESULTS

The Putative Spindle Matrix Protein Skeletor Colocalizes with the Bx34 Antigen (Megator) during Mitosis

In a search for candidate proteins that potentially could interact with the putative spindle matrix, we conducted labeling studies with the Bx34 mAb (Zimowska et al., 1997). The Bx34 antigen (Megator) previously was found to be localized to the nuclear rim and to the nuclear extrachromosomal space during interphase; however, considerable Bx34 immunoreactivity was also reported to be present around the metaphase plate during mitosis, although the nature of this labeling was not resolved (Zimowska et al., 1997). Similar labeling around the metaphase plate was also observed by Frasch et al. (1986) with several other *Drosophila* mAbs. For these reasons we revisited the issue of mAb Bx34’s labeling during the cell cycle in syncytial *Drosophila* embryos fixed with Bouin’s fluid, a precipitative fixative characterized by its rapid penetration and efficient fixation of nuclear proteins (Johansen and Johansen, 2003). As illustrated in Figure 1 the Bx34 mAb in addition to its characteristic interphase staining pattern also labeled what appeared to be fusiform spindle structures at meta- and anaphase. We observed this distribution of Megator both in Bouin’s fluid and PFA fixed preparations as well as with a polyclonal antiserum made toward a synthetic peptide based on Megator’s amino acid sequence (Zimowska et al., 1997). Although the spindle-like labeling of the Bx34 mAb was intriguing and suggested a potential colocalization with the putative spindle matrix proteins the antibody was insufficiently robust for double labeling studies. We therefore generated new Megator mAbs, 12F10 and 11E10, against a GST fusion protein containing residues 1433–1703 of the Megator protein. Both mAbs label a single protein band of \( \sim 260 \) kDa on immunoblots of S2 cell protein extracts consistent with the predicted molecular mass of Megator of 262 kDa (Figure 2A) and recapitulate the reported interphase distribution of Megator at interphase. This is shown for polytene nuclei in Figure 2B, where the Megator labeling surrounds the chromosomes labeled with Hoechst and in confocal sections of embryonic syncytial nuclei in Figure 2C where the nuclear rim labeling coincides with that of lamin antibody. We subsequently
used mAb 12F10 (IgG1) to perform double labeling studies with the Skeletor antibody 1A1 (IgM) on fixed syncytial blastoderm embryos at different stages of mitosis (Figure 3). Figure 3A shows that although Megator and Skeletor labeling are intermingled in the nuclear interior, only Megator staining is prominent at the nuclear rim. Although embryonic interphase nuclei do not afford sufficient resolution to determine whether Skeletor and Megator labeling are separate in the nuclear interior, this can be clearly demonstrated in light squashes of polytene salivary gland nuclei where Skeletor is localized on the chromosomes that are surrounded by Megator labeling (Figure 3B). However, as mitosis commences Megator reorganizes during prophase into a fusiform spindle structure the pattern of which at prometaphase and anaphase appears identical to that of the putative spindle matrix protein Skeletor (Figure 3A). At telo-

**Figure 3.** The dynamic redistribution of Megator relative to the putative spindle matrix protein Skeletor during the cell cycle. The images are from double labelings of Megator with mAb 12F10 (green) and Skeletor with mAb 1A1 (red). The composite images (comp) are shown to the left. (A) At interphase Skeletor and Megator labeling are intermingled in the nuclear interior, whereas Megator labeling is prominent at the nuclear rim. During prometa- and anaphase the composite images (comp) show extensive overlap between Megator and Skeletor labeling as indicated by the predominantly yellow color. At telophase where Skeletor begins to redistribute back to the chromosomes, Megator appears to be preferentially localized to the spindle midbody. The images are from confocal sections of syncytial embryonic nuclei. (B) Light squash of a larval polytene nucleus, where Skeletor localized on the chromosomes are surrounded by Megator labeling.
phase Skeletor begins to redistribute back to the decondensing chromosomes, whereas at this stage the majority of Megator is localized to the spindle midbody (Figure 3A).

To address the relationship between the Megator and microtubule spindles, we conducted triple-labeling studies in embryos where microtubules were disassembled by either nocodazole or cold treatment as previously described (Walker et al., 2000). Figure 4A shows an image of a Megator spindle from a cold-treated embryo arrested at metaphase (bottom panel) compared with a control labeling (top panel). In the control labeling the Megator-defined spindle and the microtubule spindle are coaligned (Figure 4A, top panel). In contrast, after cold treatment there was no detectable tubulin antibody labeling indicating complete disassembly of the microtubules (Figure 4A, bottom panel). However, even in the absence of microtubule spindles, the Megator spindle remains intact implying that the existence of the Megator spindle does not require polymerized microtubules. Furthermore, under such depolymerized tubulin conditions both Megator and Skeletor spindle labeling are present and showing extensive colocalization (Figure 4B). This suggests that both Megator and Skeletor may be contributing to the formation of a spindle-like structure, the integrity of which is largely independent of microtubules.

The spindle localization of Megator is not restricted to the early embryonic cycles of nuclear division that lack the normal cell cycle checkpoints. We analyzed Megator distribution in the S2 cell line, which is a cell line that was originally derived from later stage embryonic cells (~16 h). In these cells, Megator shows a similar distribution pattern to that of syncytial blastoderm embryos (Figure 5). At interphase Megator is present in the nuclear interior and colocalizes with lamin at the nuclear rim (Figure 5A) whereas at metaphase Megator and Skeletor are colocalized at a spindle-like structure distinct from the chromosomes congregated at the metaphase plate (Figure 5B).

**Megator Molecularly Interacts with the Putative Spindle Matrix Complex**

To address whether Megator may interact with the putative Skeletor/Chromator spindle matrix complex we performed coimmunoprecipitation experiments designed to test for molecular interactions. For these experiments proteins were extracted from *Drosophila* embryos, immunoprecipitated with Megator or Chromator antibody, fractionated on SDS-PAGE after the immunoprecipitation, immunoblotted, and probed with antibody to Chromator and Megator, respectively. Figure 6A shows such an immunoprecipitation experiment where Chromator antibody coimmunoprecipitated a 260-kDa protein that is detected by Megator antibody on Western blots. Western blot analysis also confirms that this band comigrates with Megator protein from total embryo lysate or from Megator antibody immunoprecipitation samples. In the converse experiment the immunoprecipitate of Megator antibody contained a 130-kDa band detected by Chromator antibody that was also present in the lysate and in the Chromator immunoprecipitate sample (Figure 6B). This band was not present in lanes where immunobeads only were used for the immunoprecipitation (Figure 6, A...
Megator Is an Essential Gene

Megator has been previously cloned and sequenced and encodes a large 2346 amino acid protein of 262 kDa in which the NH2-terminal 70% is predicted to form an extended coiled-coil region while the COOH-terminal 30% is unstructured and acidic (Zimowska et al., 1997; Figure 7A). It contains a putative nuclear localization signal (NLS) in the COOH-terminal part (Figure 7A). By PCR mapping and sequencing we determined that the P-element present in the l(2)k03905 line (Spradling et al., 1999) is inserted at the start of the published cDNA of Megator (Zimowska et al., 1997) at position +1 (Figure 7A). This insertion event also resulted in a 9-base pair duplication including 8 base pairs of upstream genomic sequence and a duplicated +1 residue. The site and nature of the insertion suggests that a functional Megator transcript is not likely to be made from the mutant gene and thus this insertion may represent a null mutation. To determine the viability of Megator mutants we analyzed the offspring from crosses of l(2)k03905/CyO, P[w+mc] = Act-GFP]MR1 parents in which the balancer chromosome is labeled with GFP allowing for the identification of homozygous embryos and larvae. No homozygous embryos or one homozygous l(2)k03905/l(2)k03905 first instar larvae emerged. This suggests that the Megator protein is essential and that the lethality caused by the P-element mutation largely occurs during embryonic development as maternal stores are exhausted. Consistent with this we find that Western blots (Figure 7B) of homozygous 15–20 h l(2)k03905 mutant Megator embryos show decreased Megator protein levels of only 28.5 ± 7.6% (n = 4) that of Megator levels in l(2)k03905/CyO and CyO/CyO embryos from the same embryo collection. We quantified this difference by determining the average pixel density of mAb Bx34 immunoblot staining of equal numbers of homozygous l(2)k03905 mutant Megator embryos and control embryos. The remaining low level of Megator protein observed in the homozygous mutant is likely due to residual maternal stores.

In a recent study, it was found that in a significant percentage of lethal mutant lines carrying characterized P insertions, the lethal mutation was not directly associated with the P insertion event itself (Bellotto et al., 2002). For this reason it was essential to confirm that the P insertion is the source of lethality for the l(2)k03905 allele. To address this concern, we screened for precise excision events by introducing the Δ2–3 transposase to mobilize the transposon and then selecting for loss of the mini-white marker that is carried by the P-element. Stocks established from such flies were then analyzed by PCR to characterize the nature of the excision event to identify those lines with precise excisions of the P element. Test crosses of such lines demonstrated that the precise excision of the P element restored Megator expression and viability to flies that were homozygous for the second chromosome that had previously carried the l(2)k03905 insertion (unpublished data). That precise excision of the l(2)k03905 P element in three independent lines restores Megator expression and viability supports that the lethality observed in the l(2)k03905 mutant line was directly due to the insertion of the P element in the Megator region.

Functional Consequences of Reduced Megator Protein Levels

The cross immunoprecipitation experiments and the immunolabeling results are consistent with that Megator and Chromator are present in the same macromolecular complex during mitosis. This suggests that Megator has the potential to play a functional role in proper cell division. Unfortunately, this hypothesis cannot be tested in homozygous l(2)k03905 early embryos due to the presence of maternally

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**Figure 5.** Nuclear localization of Megator in S2 cells. (A) Interphase nucleus labeled with mAb 12F10 (Megator in green), lamin antibody (red), and Hoechst (DNA in blue). The composite image (Megator/lamin) shows considerable overlap (yellow color) between Megator and lamin at the nuclear rim, whereas only Megator is detected in the nuclear interior. (B) Metaphase cell labeled with mAb 12F10 (Megator in green), mAb 1A1 (Skeletor in red), and Hoechst (DNA in blue). Megator and Skeletor labeling show extensive overlap (yellow color in the composite image [comp]) at the Skeletor defined spindle. All images are from confocal sections.
Immunoprecipitation (ip) of lysates from lane 3 and 4, respectively) but not in the control sample (lane 2). (B) and Chromator immunoprecipitation samples as a 260-kDa band of embryo lysate is shown in lane 1. Megator is detected in the Megator mAb 12F10 for detection. Megator antibody staining of mutations were analyzed by SDS-PAGE and Western blotting using (A) Immunoprecipitation (ip) of lysates from Drosophila embryos were performed using Chromator antibody (mAb 12H9, lane 4) and Megator antibody (mAb 12F10, lane 3) coupled to immunobeads or with immunobeads only as a control (lane 2). The immunoprecipitations were analyzed by SDS-PAGE and Western blotting using Megator mAb 12F10 for detection. Megator antibody staining of embryo lysate is shown in lane 1. Megator is detected in the Megator and Chromator immunoprecipitation samples as a 260-kDa band (lane 3 and 4, respectively) but not in the control sample (lane 2). (B) Immunoprecipitation (ip) of lysates from Drosophila embryos were performed using Chromator antibody (mAb 12H9, lane 3) and Megator antibody (mAb 12F10, lane 4) coupled to immunobeads or with immunobeads only as a control (lane 2). The immunoprecipitations were analyzed by SDS-PAGE and Western blotting using Chromator mAb 6H11 for detection. Chromator antibody staining of embryo lysate is shown in lane 1. Chromator is detected in the Megator and Chromator immunoprecipitation samples as a 260-kDa band (lanes 4 and 3, respectively) but not in the control sample (lane 2).

Figure 6. Megator and Chromator immunoprecipitation assays. (A) Immunoprecipitation (ip) of lysates from Drosophila embryos were performed using Chromator antibody (mAb 12H9, lane 4) and Megator antibody (mAb 12F10, lane 3) coupled to immunobeads or with immunobeads only as a control (lane 2). The immunoprecipitations were analyzed by SDS-PAGE and Western blotting using Megator mAb 12F10 for detection. Megator antibody staining of embryo lysate is shown in lane 1. Megator is detected in the Megator and Chromator immunoprecipitation samples as a 260-kDa band (lane 3 and 4, respectively) but not in the control sample (lane 2). The immunoprecipitations were analyzed by SDS-PAGE and Western blotting using Chromator antibody (mAb 12H9, lane 3) and Megator antibody (mAb 12F10, lane 4) coupled to immunobeads or with immunobeads only as a control (lane 2). The immunoprecipitations were analyzed by SDS-PAGE and Western blotting using Chromator antibody (mAb 12H9, lane 3) and Megator antibody (mAb 12F10, lane 4) coupled to immunobeads or with immunobeads only as a control (lane 2). The immunoprecipitations were analyzed by SDS-PAGE and Western blotting using Chromator antibody (mAb 12H9, lane 3) and Megator antibody (mAb 12F10, lane 4) coupled to immunobeads or with immunobeads only as a control (lane 2). The immunoprecipitations were analyzed by SDS-PAGE and Western blotting using Chromator antibody (mAb 12H9, lane 5) and Megator antibody (mAb 12F10, lane 6) performed using Chromator antibody (mAb 12H9, lane 3) and Megator antibody (mAb 12F10, lane 4) coupled to immunobeads or with immunobeads only as a control (lane 2). The immunoprecipitations were analyzed by SDS-PAGE and Western blotting using Chromator antibody (mAb 12H9, lane 3) and Megator antibody (mAb 12F10, lane 4) coupled to immunobeads or with immunobeads only as a control (lane 2). The immunoprecipitations were analyzed by SDS-PAGE and Western blotting using Chromator mAb 6H11 for detection. Chromator antibody staining of embryo lysate is shown in lane 1. Chromator is detected in the Megator and Chromator immunoprecipitation samples as a 260-kDa band (lanes 4 and 3, respectively) but not in the control sample (lane 2).

derived Megator protein which masks any potential phenotypes. Furthermore, these animals die before hatching precluding larval neuroblast analysis. For these reasons, we used RNAi methods in S2 cells to deplete Megator protein levels (Figure 8). While we did not observe any obvious perturbation phenotypes of tubulin spindle morphology or chromosome segregation defects by antitubulin and Hoechst labeling of the cells (unpublished data) the number of cells undergoing mitosis was greatly reduced in Megator RNAi treated cultures (Figure 8A). In five separate experiments we determined the mitotic index defined as the number of cells in meta- and anaphase as a percentage of total cell number. Experimental cultures had a mitotic index of 1.8 ± 0.3% (n = 5) vs. an index of 4.3 ± 0.5% (n = 5) in mock treated control cultures representing a reduction of nearly 60% of cells undergoing cell division (Figure 8A). This difference is statistically significant on the p < 0.0025 level (Student’s t test). The degree of Megator knock down in the cultures was determined by immunoblot analysis (Figure 8B) and averaged 86 ± 7% (n = 3) that of mock treated controls. These results suggest that depletion of Megator may prevent cells from entering metaphase.

The COOH-terminal Fragment of Megator Is Sufficient for Nuclear and Spindle Localization
Sequence analysis of Megator identified only one previously known domain, the extended NH2-terminal coiled-coil domain, in addition to a putative nuclear localization signal (NLS) in the COOH-terminal part. Coiled-coil domains are known to be protein-protein interaction domains that often are involved in self assembly of filamentous structures (Fuchs and Weber, 1994). We therefore tested whether the coiled-coil domain plays a role in the localization of Megator to the putative spindle matrix structure. We made four constructs containing Megator sequences for expression in S2 cells carrying a COOH-terminal V5-tag. The four constructs were a full length Megator construct (Meg-FL), an NH2-terminal construct (Meg-NT) containing sequence from the starting methionine to residue 1431 that includes 87% of the coiled-coil domain, a COOH-terminal construct (Meg-CT) from residue 1758 to the terminal proline residue containing the putative NLS motif, and a smaller middle construct (Meg-M) from residue 1432–1709. Figure 9 shows examples of expression of these constructs in transiently or stably transfected S2 cells detected with V5-antibody and double-or triple-labeled with lamin or tubulin antibody and Hoechst. The Meg-FL construct localizes to the nucleus (Figure 9A) although its overexpression often leads to aggregation. It is present at the nuclear rim in lamin double labelings at interphase (Figure 9A, top panel) and it is localized to the spindle at metaphase although the distribution is abnormal with aggregation around the spindle poles (Figure 9A, bottom panel, white arrows). The Meg-NT construct containing the coiled-coil domain is not targeted to the nucleus and remains in the cytoplasm typically forming small aggregates (Figure 9B-1). However, in ~30% of transfected S2 cells (n = 320) the Meg-NT construct forms several large spheres outside the nucleus. Three examples of this are shown in Figure 9B. Figure 9B4 shows a maximum projection image from a transfected S2 cell double labeled with Hoechst, whereas Figure 9B5 shows a single confocal section from a different cell demonstrating that the spheres are hollow. Figure 9B6 is a stereo image illustrating the spatial relationship between the spheres. These data suggest that the coiled-coil domain although not targeted to the nucleus nevertheless has the ability to selfassemble into hollow spherical structures. In contrast, the Meg-CT construct is localized to the nucleus, including the nuclear rim at interphase, and colocalizes with the tubulin spindle at metaphase (Figure 9C). Thus the localization of the COOH-terminal Megator construct during the cell cycle phenocopies that of endogenous Megator observed with Megator antibody labeling. This indicates that the coiled-coil domain is not necessary for targeting of Megator to the nucleus but rather that COOH-terminal sequences are sufficient for both nuclear and spindle localization. The Meg-M construct localizes to the cytoplasm, is not present in the nucleus, and does not appear to form aggregates (Figure 9D), suggesting that the behavior of the three other constructs are independent of the V5 tag.
DISCUSSION

In this study we show that the Bx34 antigen in addition to the previously reported localization to the extrachromosomal space and nuclear rim at interphase (Zimowska et al., 1997) also interacts with the putative spindle matrix proteins, Skeletor and Chromator, during mitosis. The organization of the Bx34 antigen with a large NH2-terminal coiled-coil domain and a shorter acidic COOH-terminal domain is similar to the structure of the mammalian Tpr (translocated promoter region) protein (Mitchell and Cooper, 1992) and like Tpr the Bx34 antigen is found at the nuclear rim, likely in association with nuclear pore complexes (Zimowska et al., 1997). However, comparison of Tpr and the Bx34 antigen sequences show a very low level of identity on the amino acid level (Zimowska et al., 1997) and although the Bx34 antigen is abundant in the nuclear interior, mammalian Tpr is restricted to the nuclear periphery (Frosst et al., 2002). Furthermore, mammalian Tpr has not been observed to localize to the spindle at metaphase. Thus, although structurally similar, there is likely to be significant functional differences between the Bx34 antigen and mammalian Tpr, wherefore we have named the Bx34 antigen in Drosophila, Megator.

The presence of a large coiled-coil domain in Megator raises the intriguing possibility that it could comprise the structural element of a potential spindle matrix. Because both Chromator and Skeletor localize to chromosomes as well as to the spindle-like structure, it was not clear whether the physical interactions observed in co-ip and pull-down experiments between these molecules reflected interactions in chromosomal complexes or interactions on the spindle-like structure or both (Rath et al., 2004). However, because Megator is not localized to the chromosomes during interphase or on centrosomes during metaphase through telophase, the molecular interaction of the complex observed likely occurs on the spindle-like structure. Interestingly, the Megator deletion construct analysis in S2 cells indicate that the NH2-terminal coiled-coil containing domain has the abil-
Figure 9. Expression of V5-tagged Megator deletion constructs in S2 cells. The expressed constructs are diagrammed beneath the micrographs. (A) Full-length V5-tagged Megator (Meg-FL) localizes to the nuclear interior and nuclear rim (arrows) of S2 cells at interphase (top panel). The cells were triple-labeled with V5-antibody to visualize the Meg-FL construct (green), lamin antibody (red), and Hoechst to visualize the DNA (blue). The bottom panel shows S2 cells at metaphase labeled with V5-antibody (green), tubulin antibody (red), and Hoechst (DNA in blue). As shown in the composite image (comp) Meg-FL labeling overlaps that of tubulin (yellow color). However, the overexpressed Meg-FL construct also show some aggregation (white arrows). (B) V5-tagged NH2-terminal Megator deletion construct (Meg-NT) truncated just before the end of the coiled-coil region localizes to the cytoplasm and is mainly absent from the nucleus (top panel). The Meg-NT construct was visualized with V5-antibody (green) and the DNA with Hoechst (blue). In 30% of S2 cells the Meg-NT construct formed several large spheres outside the nucleus (bottom panel). (B4) Maximum projection image from a Meg-NT (green) transfected S2 cell double-labeled with Hoechst (blue). (B5) Single confocal section through a transfected S2 cell demonstrating that the spheres are hollow. (B6) Stereo image of a Meg-NT–transfected cell illustrating the spatial relationship between the spheres. (C) S2 cells at inter- and metaphase expressing a V5-tagged COOH-terminal deletion construct (Meg-CT) lacking the coiled-coil domain. At interphase Meg-CT localizes to the nuclear interior and to the nuclear rim (white arrows). The nucleus was labeled with V5-antibody (green), lamin antibody (red), and the DNA with Hoechst (blue). At metaphase (bottom panel) Meg-CT colocalizes with the microtubule spindle as indicated by the yellow color in the composite image (comp). The cell was labeled with V5-antibody (green), tubulin antibody (red), and the DNA with Hoechst (blue). (D) Interphase labeling in the cytoplasm of an S2 cell expressing the Meg-M construct. The cell was labeled with V5-antibody (green), tubulin antibody (red), and the DNA with Hoechst (blue). All images are from confocal sections. On the diagrams the coiled-coil region is in black, the NLS is indicated by a black bar, and the V5-tag by a gray circle.
ity to selfassemble into spherical structures in the cytoplasm. This is in contrast to the acidic COOH-terminal domain, which is targeted to the nucleus, implying the presence of a functional nuclear localization signal. Furthermore, the COOH-terminal domain is sufficient for localization to the nuclear rim as well as for spindle localization. Thus, an attractive hypothesis is that the COOH-terminal domain functions as a targeting and localization domain, whereas the NH2-terminal domain may be responsible for forming polymers that may serve as a structural basis for the putative spindle matrix complex. Supporting this notion is the finding that Megator spindles persist in the absence of microtubules depolymerized by cold or nocodazole treatment. The localization of Megator to at least three cellular compartments (nuclear rim, extrachromosomal nuclear space, spindle matrix complex) and reorganization during the cell cycle suggest that it is highly dynamic and that it may exist in several structural forms (Zimowska and Paddy, 2002). This is underscored by the finding that 1 h after heat-shock treatment the amount of Megator protein in the extrachromosomal space diminishes, whereas accumulation occurs at a single chromosomal heat-shock puff, 93D; however, as this occurs Megator localization to the nuclear rim remains unchanged (Zimowska and Paddy, 2002).

The colocalization of Megator with the Skeletor- and Chromator-defined spindle matrix during mitosis suggests that Megator may be involved in spindle matrix function. A spindle matrix has been hypothesized to provide a stationary substrate that anchors molecules during force production and microtubule sliding (Pickett-Heaps et al., 1997). Such a matrix could also be envisioned to have the added properties of helping to organize and stabilize the microtubule spindle (Johansen and Johansen, 2002). Previously, we demonstrated using RNAi assays in S2 cells that depletion of Chromator protein leads to abnormal spindle morphology and that chromosomes are scattered in the spindle, indicating defective spindle function in the absence of Chromator (Rath et al., 2004). However, we are not able to infer a clear functional role for Megator based on the results obtained in the present study. When Megator levels are knocked down by RNAi in S2 cell cultures, the number of cells undergoing mitosis is greatly reduced. However, we did not observe any cells with obvious defects in tubulin spindle morphology or chromosome segregation defects, suggesting that depletion of Megator prevents cells from entering metaphase. This could be due to an essential function of Megator in maintaining nuclear structure and/or in maintaining the integrity of the nuclear rim and pore complexes during interphase or a necessary function for nuclear reorganization during prophase. Thus, if Megator plays multiple functional roles as its dynamic localization pattern suggests (Zimowska and Paddy, 2002), it would prevent us from analyzing a mitotic function using RNAi approaches. That Megator is an essential protein necessary for viability is supported by the embryonic lethality observed as a consequence of P insertions in the Megator gene.

Studies using preparations spanning the evolutionary spectrum from lower eukaryotes to vertebrates have provided new and intriguing evidence that a spindle matrix may be a general feature of mitosis (Bloom, 2002; Johansen and Johansen, 2002; Kapoor and Mitchison, 2001). Here we show that at least three proteins, Megator, Chromator, and Skeletor, from two different cellular compartments reorganize to form a putative spindle matrix during mitosis in Drosophila. Furthermore, the Megator and Skeletor defined fusiform spindle structure remains intact even in the absence of polymerized microtubules. The identification of several potential spindle matrix molecules in Drosophila together with P-element mutations in their genes should provide an avenue for further genetic and biochemical experiments. Especially, the future isolation and characterization of point mutations in Megator promises to provide the means to separate Megator’s role in spindle matrix function from its role at other stages of the cell cycle.

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REFERENCES


Megator Localizes to the Spindle Matrix

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