The slender lobes gene, identified by retarded mushroom body development, is required for proper nucleolar organization in Drosophila

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Abstract

The nucleolus dynamically alters its shape through the assembly and disassembly of a variety of nucleolar components in proliferating cells. While the nucleolus is known to function in vital cellular events, little is known about how its components are correctly assembled. Through the analysis of a Drosophila mutant that exhibits a reduced number of mushroom body (MB) neurons in the brain, we reveal that the slender lobes (sle) gene encodes a novel nuclear protein that affects nucleolar organization during development. In sle mutant neuroblasts, the nucleolus was packed more tightly, forming a dense sphere, and the nucleolar proteins fibrillarin and Nop60B were abnormally distributed in the interphase nucleolus. Moreover, another nucleolar marker, Aj1 antigen, was localized to the center of the nucleolus in a manner complementary to the Nop60B distribution, and also formed a large aggregate in the cytoplasm. While developmental defects were limited to a few tissues in sle mutants, including MBs and nurse cells, the altered organization of the nucleolar components were evident in most developing tissues. Therefore, we conclude that Sle is a general factor of nuclear architecture in Drosophila that is required for the correct organization of the nucleolus during development.

Introduction

The nucleolus is a nuclear compartment found in eukaryotic cells in which ribosomal synthesis takes place. Many nucleolar proteins that are implicated in transcription, pre-rRNA processing, and ribosome assembly are known to form a complex together with certain species RNA and rDNA (Olson et al., 2000). These molecules dynamically alter their state of assembly during the cell cycle; they are assembled near the end of mitosis, maintain their association during interphase, and are disassembled upon the reinitiation of mitosis. In contrast to cytoplasmic organelles such as the endoplasmic reticulum or the Golgi apparatus, nucleoli lack surrounding membranes. Therefore, certain mechanisms must exist to regulate physical interactions among nucleolar components.

The nucleolus does not simply consist of an assembly of uniformly distributed components. The nucleolus generally comprises three morphologically distinct domains: the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC). These domains correlate with distinct functions during ribosomal synthesis; rRNA transcription occurs in FC or DFC, and the late processing of rRNA is carried out in the surrounding GC (Raska, 2003). While vertebrate and plant cells show this tripartite organization, the nucleoli in Drosophila tissue culture cells are less organized (Knibiehler et al., 1982).

The dynamic assembly and disassembly of the nucleolus are coupled with cell-phase progression. A molecular
mechanism contributing to the coordination of these processes has recently been identified through investigation of the activity of cyclin-dependent kinase (CDK). The inhibition of CDK activity hampers proper pre-rRNA processing and induces disorganization of nucleoli in HeLa cells (Sirri et al., 2002). In addition, nucleolar mechanisms that can reciprocally control cell-cycle progression have been identified in studies of two nucleolar proteins: Arf, which sequesters MDM2, a negative regulator of p53, into the nucleolus (Weber et al., 1999), and nucleostemin, which interacts with p53 and regulates the proliferation of CNS cells (Tsai and McKay, 2002). Thus, nucleolar mechanisms processing and induces disorganization of nucleoli in Hela inhibition of CDK activity hampers proper pre-rRNA of the activity of cyclin-dependent kinase (CDK). The processes has recently been identified through investigation mechanism contributing to the coordination of these other roles in the induction of nuclear protein export (Zolotukhin and Felber, 1999) and the sequestration of ribosomal synthesis and cell-cycle control, nucleoli have been identified in studies of two nucleolar proteins: Arf, which sequesters MDM2, a negative regulator of p53, into the nucleolus (Weber et al., 1999), and nucleostemin, which implies that the proper organization of nucleoli is crucial for normal development, and further highlight the importance of molecular mechanisms that regulate dynamic nucleolar organization.

Recently, it has been reported that the maternal-effect factor NPM2 in mice is critical for nuclear and nucleolar organization and early embryonic development (Burns et al., 2003). The Xenopus germ cell proteins FRGY2a and FRGY2b have been shown to reversibly disassemble somatic nucleoli in egg cytoplasm (Gonda et al., 2003). These studies were initiated to further the understanding of the chromatin remodeling that occurs upon fertilization as well as that of the disassembly of nucleoli upon somatic nuclear transplantation in animal cloning. The fundamental question of how the nucleolus is properly organized during the cell cycle has been approached primarily through the study of oocytes and early embryos. New approaches are necessary, to identify and analyze as yet unknown factors, in order to understand the underlying mechanisms and possible regulatory cascades involved in nucleolar organization.

In this study, we have identified the slender lobes (sle) gene in Drosophila melanogaster through the analysis of flies that carry a mutation in this gene and consequently display a reduction in proliferation of the MB neuroblasts. Our data indicate that the Sle protein, which is enriched in the perinucleolar area of the interphase nucleus, is required for the proper organization of the nucleolus in most developing cells.

Materials and methods

Fly strains

We identified GS 3144 among the lines generated by the insertion of a composite P element containing UAS sequences, as previously described (Toba et al., 1999). OK107-GAL4 was used to label adult MB neurons (Connolly et al., 1996), and UAS-mCD8::GFP (Lee and Luo, 1999) was employed as a marker to visualize cell membranes and axons. We found that ap-GAL4 labels larval and pupal MB neuroblasts and subsets of surrounding neurons that extend the axons to MB lobes.

Generation of deficiency strains and genomic rescue

Two sle-deficient lines, sle057 and sle256, were generated from GS3144 by the imprecise excision of the P element. These two lines display similar phenotypes. DNA breakpoints were examined using PCR to amplify genomic DNA extracted from homozygous flies, followed by sequencing of the relevant genomic segments. To rescue Sle expression in the mutants, an 11-kb EcoRI fragment (see Fig. 1A) was subcloned from the BAC clone R03L12 (BACPAC Resources), which contains the 86A2 region, into pCasPER. This plasmid was injected into early embryos according to the standard procedure for P-mediated germline transformation. The transformants were then crossed to mutants and examined for recovery of wild-type phenotypes.

Antibody production

To express a protein fragment to use as an antigen, we subcloned a 0.6-kb SmaI–XhoI fragment of the EST clone GM08848 into pGEX6P-3, producing pGEX-SX. To produce a second antigenic fragment, a 0.6-kb HindIII–BglII fragment from the third exon of sle was subcloned from the BAC clone R03L12 into the HindIII–BamHI site of BS II. A Sall–NotI fragment from this plasmid was then inserted into the Sall–NotI site of pGEX6P-2, to obtain pGEX-HG. The GST-fusion proteins were then expressed in the BL21 strain, purified with glutathione-beads, and used to immunize rabbits for antibody production.

Immunohistochemistry and microscopy

Dissected tissues were fixed in 4% paraformaldehyde for 20 min to 2 h. After washing twice in PBS and several times in PBT (PBS with 0.5% Triton X-100), samples were incubated with primary antibodies in PBT at 4°C. The preparation of embryos was performed following standard protocols. We used the following primary antibodies in this study: rabbit anti-Sle SX and HG (1:200–500), rat anti-mCD8 (1:200–500; Caltag), mouse anti-Dac2–3 (1:20–100; DSHB), rabbit anti-Deadpan (1:500; Bier et al., 1992), mouse anti-fibrillarin 72B9 (1:200; Cytoskeleton Inc.), mouse anti-lamin Dm0 611A3A6 (1:2; Ulitzur et al., 1997), mouse Aj1 (1:2 or 1:40 for co-staining with anti-lamin; Frasch et al., 1986), mouse anti-BrdU BMC9318 (1:20; Roche), rat anti-BrdU...
BU1/75 (1:200; Abcam Limited), rat anti-α Tubulin YL1/2 (1:100–500; Chemicon), and rabbit anti-PH3 (1:400; Upstate). We used secondary antibodies conjugated to Alexa 488, 594, and 647 (1:200; Molecular Probes) for the detection of antibody binding. TOTO-3 (1:400; Molecular Probes) was used to stain nuclei following RNase A treatment (2 mg/ml). HRP-conjugated anti-rat IgG was employed to quantitate BrdU-positive cells. Individual neuroblasts and their progeny were observed using a Leica SP2 laser scanning microscope with a 63× water-immersion object lens and a Zeiss Axiophoto 2 microscope with a 63× oil-immersion object lens and Nomarski optics. Obtained images were processed using Adobe Photoshop. Preparation for EM analyses was performed as previously described (Matsumoto et al., 1988).

**BrdU incorporation**

Proliferating cells from whole-mount larval and pupal brains were detected by BrdU incorporation. Dissected brains from staged larvae or pupae were initially transferred to Schneider medium (Sigma) that contained BrdU at a concentration of 75 μg/ml and incubated for 2 h. These tissues were rinsed in PBS, fixed in 4% paraformaldehyde for 1 h, and pretreated with 2 N HCl for 30 min. The samples were then incubated with Rat anti-BrdU BU75/1 Mab to detect BrdU-positive cells and with mouse anti-Dac

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**Fig. 1. Genomic organization of slender lobes and the mutant phenotypes of sle alleles.** (A) The transcription unit of sle (exons displayed in black boxes) is shown in its genomic environment near cytological region 86A8–B1 (The FlyBase Consortium, 2003). The P element (arrow with triangle) in line GS3144 (Toba et al., 1999) was inserted 8 bp upstream of the putative transcription start site. The start codon (ATG) is located 107 bp into the first exon. The sequences deleted in sle057 and sle256 are indicated in parentheses. The 11-kb EcoRI fragment encoding sle that was used for rescue experiments is shown at the bottom. (B and C) Mushroom body (MB) in the adult brain labeled with OK107-Gal4/UAS-mCD8::GFP. Wild-type MBs (B) contain lobe structures, a cell body cluster (asterisk), and a calyx. α, β, and γ designate the α, β, and γ lobes, respectively. Arrows indicate the diameter of the α lobe. sle057 MBs (C) have thinner α and β lobes. The β lobes are fused in the middle of the mutant brain (arrowhead). Scale bars, 50 μm. (D and E) Clusters of MB cells in the pupal brain of a wild-type (D) and a sle057 mutant (E). The number of MB neurons labeled with anti-Dac (green) is highly reduced in the mutant. There are four clusters of cells positive for BrdU incorporation (magenta) in both the wild-type and mutant MBs. Scale bars, 20 μm.
to label MB neurons, allowing the identification of MB neuroblasts.

Flow cytometry

Wing discs or central brains were dissected in PBS from eight wandering third instar larvae reared in a corn meal-EBIOS medium. Samples were incubated in 0.5 ml of Trypsin-EDTA (0.25% Trypsin, 1 mM EDTA, Gibco) with 2 µg/ml Hoechst 33342 for 2 h. Dissociated cells were sorted by an Epics Altra cell sorter (Beckman Coulter) and analyzed by EXPO32 software.

Results

Identification of the slender lobes gene

We identified the slender lobes (sle) gene from a viable P-element insertion allele created during an insertional mutagenesis screen that used lines previously developed for the gene search system (Toba et al., 1999). The P-element insertion was found 8 bp upstream of a transcription unit, CG12819-RB, predicted from several cDNA sequences (The FlyBase Consortium, 2003). Mobilization of the transposon generated two deletion alleles, sle057 and sle256, which removed genomic segments from −150 bp to 1535 bp and from −1437 bp to 955 bp, respectively (Fig. 1A). The sle256 allele contained a deletion of an adjacent putative gene, CG12818, but in sle057, only a short upstream regulatory region and a portion of the transcribed sle sequence were removed. Both deficiencies extended into the sle open reading frame, probably resulting in protein null alleles (described below).

Both male and female flies homozygous for both alleles were viable, but sterile. These animals developed more slowly, emerging from the pupal case 1 day later than wild-type animals. No obvious mutant phenotypes were detected upon examination of the external morphology of the adult body and brain. However, we found that the mushroom body (MB), a functional center for learning and memory, clearly exhibited abnormally thin axonal lobes (Figs. 1B–E). The adult MB contains five axonal lobes (α, β, α′, β′, and γ) and a dendritic cluster called a calyx (Crittenden et al., 1998). MBs in the adult mutant, labeled with a membrane-tethered GFP driven by a MB-specific driver OK107-Gal4 (Connolly et al., 1996), exhibited thinner α and β lobes (Fig. 1, compare B with C). In addition, one pair of mutant β lobes was frequently found fused to each other at the center of the brain (Fig. 1C). The other lobe structures were not easily distinguishable from those in the wild type. The observed size reduction of the mutant MB could be explained by a decrease in neuronal number. To confirm this possibility, we directly quantitated the number of MB neurons in images taken with a laser-scanning microscope (LSM), and found that the mutant MB produced, on average, about half the normal number of neurons (Fig. 1, compare D with E). All aspects of the mutant phenotype, including sterility, that resulted from both alleles were successfully rescued by a sle-containing 11-kb genomic fragment (Fig. 1A) introduced by germline transformation. Therefore, we concluded that the mutant phenotype was caused by the loss of sle function.

sle MB neuroblasts fail to accelerate their proliferation

While most other neuroblasts divide during short developmental windows, MB neurons are generated from four self-renewing neuroblasts that continuously proliferate during postembryonic development (Ito and Hotta, 1992). An MB neuroblast asymmetrically divides to produce a series of ganglion mother cells (GMCs), each of which divides into two neurons. To investigate the reason for the reduction in neuronal number in the mutant MB, we examined the proliferation of MB neuroblasts by means of BrdU incorporation at the larval and pupal stages. The mutant MB displayed four clusters of labeled cells (Fig. 1E) in each brain hemisphere, as did the wild-type MB (Fig. 1D), indicating that the normal number of MB neuroblasts had differentiated, and that they all produced daughter cells. The neuroblast marker protein Deadpan (Bier et al., 1992) was consistently detected in one cell in each cluster in both the wild-type and mutant MBs (data not shown). The sle neuroblasts, however, were significantly smaller in both total cellular and nuclear size and produced fewer GMCs and neurons (Figs. 1E, 2, compare A with B). A developmental profile of BrdU incorporation (Fig. 2D) revealed that the initial proliferation rate of sle MB neuroblasts, at the first instar larval stage [1 day after egg laying (AEL)], was indiscernible from that of the wild type, in which 14 progeny cells per brain incorporated BrdU for 2 h. As development proceeded, however, wild-type neuroblasts rapidly accelerated their proliferation rate, which reached a peak (38 BrdU+ cells) at the early pupal stage (6.5 days AEL). In contrast, the proliferation of sle neuroblasts increased only minimally (20 BrdU+ cells at most), then decreased prematurely after 5 days AEL and ceased earlier than wild type by 9 days AEL. This reduced proliferation rate was associated with a failure in cellular and nuclear growth throughout development. These mutant phenotypes were also rescued by the introduction of the 11-kb genomic DNA encoding sle into the mutant (Figs. 2C,D). Therefore, these data indicate that sle is required for MB neuroblasts to accelerate their proliferation and cell growth during postembryonic neural development.

Sle functions in general cellular mechanisms rather than a specific cell-cycle control

The observed reduction in the proliferation rate of sle neuroblasts indicated an extension of cell-cycle length in
the mutant. Since it is known that cell growth regulates the progression of interphase in Drosophila wing imaginal disc cells (Prober and Edgar, 2000), the impaired cell growth of *sle* neuroblasts implied that interphase had become extended. To investigate the cell phase during which *sle* primarily functions, we stained MB neuroblasts for phosphohistone H3 (PH3), a marker that labels chromosomes in most mitotic phases. We quantitated the number of MB neuroblasts stained positively for a PH3 signal and compared the ratio of positive to negative cells in both the mutant and wild type. Wild-type animals exhibited a ratio of 0.36 (n = 216), while mutant animals possessed a ratio of 0.35 (n = 204), indicating that the relative lengths of interphase and mitosis did not change in mutant neuroblasts. These data together with the decreased proliferation rate as shown by BrdU incorporation into mutant neuroblasts indicate that the progression of both interphase and mitosis is retarded in the mutant, suggesting that *sle* functions in a more general cellular mechanism, rather than in a specific cell-cycle control.

In addition, we examined the cell cycle of wing imaginal disc cells by FACS since the mutant disc cells exhibited defects at the ultrastructural level (see later). We found that the ratios of the cells that were assigned to either G1, S, or G2/M were not significantly altered between the wild type and mutant at the third instar larval stage (data not shown). Therefore, *sle* is not likely to participate in a specific cell-cycle control in wing disc cells. Taken together, our results support the idea that *sle* is involved in general cellular mechanisms.

**The Sle protein is localized in the perinucleolar region during interphase**

The *sle* protein, predicted to be 1430 amino-acid residues in length, is rich in charged and polarized residues throughout its length. This composition results in a highly hydrophilic protein (Fig. 3B), with an abundant number of acidic residues (pl = 5.14; 294 acidic aa/1430 total aa) and a glutamate-rich region in the middle of the protein (Figs. 3A,C). We were not able to identify any apparent functional domains except for several putative nuclear localization signals and PEST sequences (Fig. 3A). In addition, *sle* contains a number of predicted α-helices, but has no clear coiled-coil structure. We compared the amino-acid sequence of *Sle* with other proteins in yeast and vertebrate, but no proteins showed significant sequence similarity.

To further examine *Sle* function, we raised anti-*Sle* antisera using two independent polypeptides, SX and HG, as antigens (Fig. 3A). The two antisera showed essentially the same staining patterns, but we predominantly employed the SX antiserum because it provided more robust signals than the HG antiserum. *Sle*-positive signals were observed in the nuclei of all examined cell types, including neuroblasts, GMCs, neurons, imaginal disc epithelial cells, germline cells, and embryonic cells. While the signal intensity varied, strong signals were generally observed in cells with high proliferation rates or protein/RNA production levels, such as neuroblasts (Fig. 3D) and nurse cells. In the brain, neuroblasts, GMCs, and young neurons exhibited stronger signals than the surrounding older neurons at many developmental time points (data not shown). In addition, *Sle* was hardly detected at all in neurons of 3-day-old adult brains. No staining was observed in *sle* mutants (Fig. 3F), but wild-type staining patterns were restored upon the introduction of the 11-kb *sle*-coding genomic fragment into the mutants (Fig. 3G), demonstrating that the antiserum is specific to the *Sle* protein and that *sle*057 and *sle*256 are protein null alleles.

Interestingly, the *Sle* protein frequently exhibited an uneven distribution pattern in the nuclei of interphase neuroblasts (Fig. 3D). The anti-*Sle* antibody labeled...
Fig. 3. Sle is a highly charged acidic nuclear protein that is enriched in the perinucleolar region. (A) Predicted Sle protein structure. The green region represents the glutamate-rich region. The black boxes beneath the bar indicate putative PEST sequences. The brown dots above the bar indicate the positions of the nuclear localization signals. The amino-acid sequence is provided at GenBank accession number AAF54522. SX and HG denote the regions of the protein used to raise antisera. (B) Kyte–Doolittle hydropathy plot. Sle is highly hydrophilic throughout its length. (C) Amino-acid profile. Boxes mark sequences containing more than five aspartate or glutamate residues (red), more than five lysine or arginine residues (blue), or more than seven aspartate, glutamate, lysine, or arginine residues (black) within a 20 amino-acid stretch. (D–G) Staining of a neuroblast (D) and neuron (E) of wandering third instar larvae for Sle (Sle, green), Fibrillarin (Fib, red), and DNA (TOTO-3, blue). Sle protein staining surrounds a fibrillarin-rich region representing the nucleolus. These signals are excluded from heterochromatin. (F) No signal could be detected in neuroblasts stained for Sle from sle mutant brains. (G) Sle staining was recovered in the sle mutant upon introduction of the sle-encoding 11-kb genomic fragment. Scale bars, 2 μm.
nucleoplasm in a network-like pattern and also revealed a high level of Sle surrounding small regions within these nuclei. Double staining for Sle and fibrillarin, a nucleolar protein involved in pre-rRNA processing that is associated with the dense fibrillar component (DFC) of vertebrate nuclei, revealed that each region surrounded by Sle was a nucleolus. Nop60B, another nucleolar protein that shares a high degree of sequence identity with yeast cbf5 involved in rRNA synthesis (Phillips et al., 1998), colocalized with fibrillarin (data not shown; see also Fig. 6), confirming that Sle is localized to perinucleolar regions within the nucleus. In addition, Sle was distributed throughout the extrachromosomal domain. We stained MB neuroblasts for Sle and DNA (with TOTO-3) and frequently observed Sle signals flanking heterochromatin (Fig. 3D). Moreover, in nurse cells during growing stages in which polytene chromosomes are present within large nuclei (Dej and Spradling, 1999), Sle was distributed throughout a region mostly complementary to the area occupied by the chromosomes (data not shown). Thus, Sle is characterized as a nuclear protein frequently enriched around the nucleolus and excluded from the chromosomal domain. Similar staining patterns in which Sle was found to surround nucleoli were observed in postmitotic neurons (Fig. 3E) and many other types of cells, suggesting that a possible role for Sle in nucleolar function or formation.

The distribution of Sle in neuroblasts, however, changed dynamically over the course of the cell cycle. Even during interphase, the signal surrounding the nucleolus varied in intensity (compare Fig. 3D with Fig. 4A). Upon entering prophase, the network-like pattern of Sle staining was lost, and the staining pattern became more evenly distributed throughout the nucleus, although Sle was still excluded from the chromosomal domain (Fig. 4B). In metaphase, Sle behaved like a spindle matrix component (Walker et al., 2000) and localized to a region surrounded by the inner spindle (Fig. 4C).

**Nucleolar organization is altered in sle mutant neuroblasts**

The assembly pattern of the nucleolus changes dramatically during the cell cycle. Staining of wild-type MB neuroblasts for fibrillarin revealed four staining patterns that reflect the altering organization of nucleolar components: ‘amorphous’, ‘solid’, ‘hollow’, and ‘negative’ (Figs. 5A–D). These patterns appeared at frequencies of 28, 33, 4, and 35%, respectively (Fig. 5H). In contrast, 50% of the nucleoli in sle neuroblasts were of the ‘hollow’ type (Figs. 5E–H). Furthermore, the ‘hollow’ nucleoli in sle mutants were large in size (Fig. 5, compare C with E and also see Fig. 5I), despite the reduced cell and nuclear sizes of sle neuroblasts. These results suggest that sle nucleoli have been shifted to an unusual assembly state, which only rarely or transiently occurs in wild-type cells during the cell cycle. The results observed for MB neuroblasts were similar to patterns exhibited by other neuroblasts in the central brain of third instar larvae and early pupae (Fig. 5E).

To further analyze the nucleolar organization in the mutant neuroblasts, we employed another nucleolar marker, the antibody Aj1 (Frasch et al., 1996). This antibody labels the nucleoli in embryos, imaginal disc cells, and salivary glands (Perrin et al., 1998). However, in the neuroblasts of the wild-type brain during interphase, we found that Aj1 only occasionally stained the nucleolus but usually labeled the entire nucleus as well as the cytoplasm in a granular pattern. When the nucleolus was stained, the Aj1 pattern roughly overlapped with the Nop60B distribution (Fig. 6A). Interestingly, in the mutant neuroblasts, Nop60B staining appeared in the hollow pattern observed for fibrillarin, and the Aj1 antigen was localized to the center in a complementary pattern. In addition, although the granular pattern was less noticeable in mutant nuclei, large aggregates containing Aj1 antigen were frequently observed in the neuroblasts that displayed the hollow nucleolar pattern (68%, n = 165; Fig. 6B). Co-staining with an anti-lamin antibody revealed that the aggregation occurred in the cytoplasm (Fig. 6C). These data indicate that nucleolar components are abnormally assembled in mutant neuroblasts; they are segregated into at least two distinct layers within the nucleolus and also undergo ectopic aggregation in the cytoplasm. Sle is therefore required for the proper organization of nucleolar components in the nucleus and also to prevent ectopic aggregation in the cytoplasm.
We next performed ultrastructural analyses using an electron microscope (EM) to further evaluate the difference in nucleolar architecture between wild-type and sle neuroblasts. Because the nucleolar assembly patterns of MB neuroblasts did not differ from other neuroblasts in either wild-type or mutant animals, we examined the nucleoli of all neuroblasts, which we identified as large cells adjacent to clusters of neurons. Our analyses revealed two forms of nucleolar morphology in wild-type neuroblasts. In the first form, the nucleolar components were assembled at a moderate packing density, and the nucleolar surface was roughly organized or tufted (Fig. 7A). This form probably corresponds to the ‘solid’ fibrillarin pattern observed under LSM. In the second form, the outline of the nucleolus was discontinuous, and it lacked the round shape of the solid pattern (Fig. 7B). In this form, two groups of components with different electron densities and morphologies were intermingled with each other, and were thought to be equivalent to the nucleoli observed under LSM to have an amorphous fibrillarin pattern. In contrast, the majority of nucleoli in sle neuroblasts exhibited a distinct assembly state. The nucleolar components were tightly organized, and the nucleolar surface was smooth, creating a dense sphere (Fig. 7C). We believe that this structure represents nucleoli with a ‘hollow’ staining pattern due to its high frequency of occurrence in mutant cells. However, the central region that remained unlabeled by fibrillarin but stained for AJ1 was packed with electron-dense materials. These findings indicate that different sets of nucleolar

Fig. 5. Altered nucleolar morphology in the sle mutant. (A–E) MB neuroblasts and their progeny from third instar larvae carrying an ap-Gal4/UAS-mCD8::GFP construct that drives membrane-tethered GFP expression (green) in MB cell lineages. Nucleoli were stained with an anti-fibrillarin antibody (magenta). Wild-type nucleoli exhibited a variety of fibrillarin staining patterns, including (A) ‘amorphous’, (B) ‘solid’, (C) ‘hollow’, and (D) ‘negative’ staining types. In sle mutants (E), all nucleoli illustrated here (arrowheads) exhibited ‘hollow’-type staining. Three of the cells shown here are MB neuroblasts (green), while the two are other neuroblast subtypes. The nucleoli in the mutant were enlarged, despite the small cell size observed in the mutant. Scale bar, 10 μm. (F and G) Nuclei of stage 7 nurse cells. In wild-type animals (F), fibrillarin signals (Fib, magenta) were scattered in spaces devoid of polytene chromosomes (DNA, stained with TOTO-3, green) throughout the nucleus. In sle nurse cells (G), fibrillarin staining often outlined a large nucleolus (arrowheads). Scale bar, 5 μm. (H) Proportion of nucleolar types defined as A: amorphous, S: solid, H: hollow, and N: negative. The assignment of nucleolar types was performed in a genotype-blind fashion. The majority of nucleoli in sle neuroblasts are ‘hollow’ in type. Small nucleoli with diameters less than 2 μm were grouped into the ‘negative’ category. n = 216 for wild type and n = 204 for sle. (I) A histogram indicating the distribution of nucleolar size in the wild-type (black) and sle (gray) MB neuroblasts. This chart confirms the increase in the number of large nucleoli in sle neuroblasts. The yellow bars represent the proportion of ‘hollow’-type nucleoli in each nucleolar-size category. The size of each nucleolus was calculated as the square root of XY, where X and Y were the longest distances measured across each nucleolus along the x and y axes, respectively. The distance was measured on merged images of three optical sections taken at 1-μm intervals.
components were tightly packed into distinct compartments in the mutants.

Our data from both LSM and EM analyses indicate that Sle is necessary for the proper assembly of nucleolar components. However, since the organization of nucleoli depends on the cell-cycle phase, it is possible that Sle more directly regulates the cell cycle, rather than the nucleolar event. To evaluate how Sle is involved in nucleolar organization, we examined the fine nucleolar structure of neurons, which are quiescent with respect to mitosis and DNA replication. The shapes of nucleoli observed in wild-type neurons from third instar larvae and early pupae were variable, and two groups of components with distinct electron densities were often closely associated with each other (Fig. 7D). In contrast, most nucleoli in sle neurons formed spheres consisting of apparently uniform components (Fig. 7E) and showed little contact with another cluster of components, even when they were located in close proximity (data not shown). The presence of abnormal nucleoli in mitotically quiescent cells suggests that Sle participates directly in the nucleolar event and only secondarily affects cell-cycle progression in mitotically active cells.

Sle is a general factor involved in nucleolar organization

Female sterility is one of the phenotypes observed in sle mutants. This could be caused by defects in the nucleoli of germline cells during oogenesis. We found that the mutant ovaries frequently contained abnormal nurse cells that were irregularly shaped. This phenotype became more apparent as the ovary development proceeded, and many nurse cells were found to collapse by stage 9 (Supplementary Fig. 1 in Appendix A). We therefore examined the morphology of nucleoli by LSM in the mutant ovaries. It has been reported that the nucleolus of a nurse cell is peculiar in shape; it is organized in a three-dimensionally complex network pattern, and it becomes more extensively distributed throughout the nucleus as the nurse cells increase their nuclear volume during oogenesis (Dapples and King, 1970). In the nurse cells of wild-type animals, fibrillarin was dispersedly distributed throughout the nucleus (Fig. 5F), reflecting the complex shape of the nucleolus. In contrast, the nucleolus of the sle nurse cell was organized into a large mass with a smooth outline; fibrillarin accumulated around the rim of the nucleolar mass, creating a ‘hollow’ region in optical
sections (Fig. 5G), as observed for the nucleoli of sle neuroblasts.

While most tissues in the mutant animals did not exhibit apparent morphological phenotypes under LSM, EM analyses unveiled abnormalities in nucleolar structure in multiple types of cells. Wing imaginal disc cells of mutant third instar larvae exhibited an altered nucleolar assembly pattern similar to that observed in neuroblasts and neurons (Figs. 7F,G). Therefore, Sle appears to be a general factor required for the proper organization of nucleolar components in developing Drosophila cells.

Discussion

Identification of a gene required for the proper nucleolar organization

In this study, we have performed a Drosophila mutant screen to identify a novel perinucleolar protein, encoded by the sle gene, that is necessary for the proper organization of nucleolar components. The Sle protein is expressed in cells of most developing tissues, including neuroblasts, imaginal disc cells, and germline nurse cells, all of which exhibited an altered nucleolar structure in the mutant. Sle may be, therefore, a general factor required for the proper organization of the nucleolus. Since ribosomal synthesis and maturation occur in the nucleolus, we examined the major rRNA species in the central nervous system and imaginal disc cells of wandering third instar larvae (unpublished data). However, we could hardly detect any alterations in both the amount and processing of rRNA in sle mutant tissues. Nevertheless, the altered nucleolar organization implies that the maturation of ribosomes may be retarded in the mutant, and the presence of Sle in the nucleus may result in the facilitation of this maturation.

Although we identified sle from the mutant phenotype in the MB, similar gross phenotypes were not observed in most tissues including the whole brain. It was surprising that the mutant brain size was apparently normal at the adult stage, because most dividing neuroblasts displayed altered nucleolar structures in the mutant brain. We presume that the neuroblasts outside MB may also proliferate at a decreased rate as do MB neuroblasts, but a lengthened proliferation...
period may sufficiently compensate the neuronal number to the wild-type level. This is possible for most neuroblasts since they divide during relatively short time windows in the wild type, contrasting to MB neuroblasts that proliferate throughout most developing stages. Alternatively, the loss of Sle function may have more pronounced effects on MB neuroblasts, which could possess particular cellular properties. For example, the MB neuroblast is one of the largest cells in the brain, with notably rapid cell division and growth that allow it to generate a large number of neurons. This high cellular activity may impose a critical requirement for Sle, in order to allow rapid and dynamic nucleolar assembly.

It would be noteworthy that the unique ability of neuroblasts to self-renew may require specific mechanisms that regulate their proliferation. It has been shown that cell proliferation in neural stem cells is controlled by the nucleolar protein, nucleostemin (Tsai and McKay, 2002). A similar association may exist between Sle function and neuroblast proliferation in Drosophila, where impaired proliferation follows from the nucleolar lesion in the sle mutant. Although the mechanisms involved in the generation of the mutant phenotype remain to be clarified, the perturbed nucleolar organization can account for the defects that we observed in cell growth, cell-cycle progression, and cell proliferation of MB neuroblasts.

The sle mutation changes the organization of nucleolar components

Vertebrate cell nucleoli comprise three subregions: the fibrillar center, the dense fibrillar component (DFC), and the granular component (GC). Drosophila nucleoli in tissue culture cells, however, are ultrastructurally less organized (Knibiehler et al., 1982). Our EM observation has confirmed this structural property in many types of cells in larvae, pupae, and adults. However, we occasionally found that a small fraction of neurons in late pupal and adult brains contained nucleoli with an electron-translucent space at the center, surrounded by an area of high electron density, followed by the outermost components (unpublished data), in a manner resembling the tripartite structure of nucleoli in vertebrates. Therefore, similar sets of components may be present in vertebrate and Drosophila nucleoli, but they are usually organized in dissimilar patterns.

In sle mutant cells, the nucleolar components are frequently organized into an electron-dense sphere, while in the wild type they appear to more dynamically change their relative positions generating variable nucleolar shapes. These observations demonstrate that the Sle protein contributes to the maintenance of a malleable nucleolar structure. In addition, while in the wild-type nucleolus the distributions of fibrillarin, Nop60B, and Aj1 antigen display an overlapping or, more locally, intermingling pattern, in sle mutant nucleoli, Nop60B and Aj1 form a concentric distribution where Nop60B segregates to the outer region and Aj1 is found in the core. Moreover, a large amount of the Aj1 antigen is aggregated in the cytoplasm. All of these data are consistent with the idea that Sle negatively modulates the selective aggregation of a subset of nucleolar components that includes the Aj1 antigen and maintains all the components in their proper arrangement at a moderate density (Fig. 8).

While the Drosophila nucleoli are less organized than the vertebrate nucleoli, we have found that two nucleolar compartments with distinct electron density were separately but closely positioned in neuroblasts possibly during interphase (Fig. 7B) and in neurons that are mitotically quiescent (Fig. 7D). Since the electron-dense compartment (g in Figs. 7B,D) contains more granular materials and the other (f in Figs. 7B,D) appears to be more fibrous, these compartments may correspond to GC and DFC defined in vertebrates, respectively. In the sle mutant cells, however, we could find a single nucleolar mass (Figs. 7C, E) that retains a staining of fibrillarin, a vertebrate DFC marker, in the structure (Fig. 5E). In addition, Sle distributes around the fibrillarin-positive region in the wild-type cells. These observations raise two possibilities; firstly, Sle may be a component of GC, and secondly, Sle is a perinucleolar factor required for the proper organization of GC in the nucleolus. The loss of Sle may cause GC to disperse in the nucleoplasm or to be incorporated into DFC, and could consequently affect the DFC organization.

How is Sle involved in nucleolar organization?

The nucleolus is a nuclear compartment that lacks a surrounding membrane. Therefore, to form a large complex, all the components must assemble correctly through physical interactions. There are many nucleolar proteins that function in pre-rRNA processing, ribosomal assembly (Olson et al., 2000), and RNA polymerization. Many of these proteins have basic domains that have been implicated in potential interactions with pre-rRNA or rDNA within the nucleolus.
nucleoli. One possible mechanism for the regulation of nucleolar assembly and disassembly is the participation of proteins with particular physical properties that either promote or inhibit protein–RNA, protein–DNA, or protein–protein intermolecular interactions. We have shown that Sle is highly charged and abundant in acidic amino-acid residues, and contains a glutamate-rich region in the middle of the protein. A stretch of glutamate residues in NPM2 has been implicated in histone and protamine binding, which facilitates nucleosome assembly and the replication of the paternal genome (Burglin et al., 1987; Dilworth et al., 1987; Dingwall et al., 1987; Ohsumi and Katagiri, 1991; Phillips et al., 1998). This line of reasoning suggests that the acidic amino-acid sequences and glutamate-rich region in Sle may be of importance in regulating nucleolar assembly and disassembly, possibly through interactions with nucleolar proteins that contain basic domains. Such physical interactions among charged proteins and nucleic acids may provide the foundation for nucleolar formation without membranes, and Sle, a nuclear protein frequently enriched around the nucleolus, could modulate these physical interactions during the cell cycle. The dynamic Sle distribution pattern as well as the selective aggregation of the Aj1 antigen in the sle mutant suggest that Sle may be directly associated with subsets of nucleolar proteins during transport from the cytoplasm to the nucleolus and may prevent their aggregation in the cytoplasm as well as in the nucleus. This association may occur immediately after de novo protein synthesis or upon redistribution of the nucleolar components during the cell cycle. Thus, without Sle, the nucleolus becomes disorganized and loses the activity required for high levels of proliferation, as observed in MB neuroblasts. In order to further understand Sle function, it will be critical to investigate the proteins that directly interact with Sle, and how these proteins become properly incorporated into the nucleolus.

In this study, we have demonstrated that Sle is required for the proper organization of nucleolar components and dynamically changes their distribution pattern within the nucleus. Further analyses of Sle will provide valuable insight into how the nucleolus, a vital apparatus for numerous cellular processes, can properly assemble and how the nucleolar organization affects cell proliferation during development.

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Appendix A. Supplementary data


References


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