Distribution of a nuclear envelope antigen during the syncytial mitoses of the early *Drosophila* embryo as revealed by laser scanning confocal microscopy

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Summary

The changing distribution of a nuclear envelope antigen recognized by a monoclonal antibody raised against human fibroblast vimentin during the syncytial mitoses of the *Drosophila* embryo has been studied with a confocal laser scanning microscope. The antigen appears very early as irregular aggregates in the peripheral cytoplasm of the preblastoderm embryo. As the first nuclei reach the periplasm the antigen is localized on the nuclear envelope and the cytoplasmic staining decreases. In addition to the perinuclear labeling we observed intense midzone and polar staining during the mitotic cycle. A possible relationship between polar localization of the antigen and centrosome position is discussed.

Key words: nuclear envelope, *Drosophila* embryo, confocal microscope.

Introduction

The onset of mitosis in higher eukaryotes is accompanied by the fragmentation of the nuclear envelope into large cisternae and the disappearance of pore complexes (Robbins and Gonatos, 1964; Roos, 1973; Zatsepina et al., 1977). This general model of nuclear envelope rearrangement, termed open mitosis (Franke, 1974), shows remarkable differentiation in the syncytial embryo of *Drosophila*. Electron microscopy revealed that the nuclear envelope remains essentially intact during mitosis of the syncytial blastoderm and large openings appear at the poles, through which the spindle microtubules run. Nuclear pore complexes are lost during mitosis and a second layer of fenestrated cisternae joins the original cisternae to form a double fenestrated layer (Stafstrom and Staehelin, 1984). Despite this structural difference in nuclear envelope organization from that of most higher organisms, polypeptides homologous to vertebrate lamins have been described in *Drosophila* embryos (Risau et al., 1981; Fisher et al., 1982; Smith et al., 1987). Immunofluorescence observations showed that the distribution of these nuclear envelope proteins is similar to that observed in vertebrate cells at mitosis, but during the rapid divisions in the early *Drosophila* embryo these antigens are never completely dispersed throughout the cytoplasm (Fuchs et al., 1983).

To follow nuclear envelope dynamics during the syncytial mitoses that precede cellularization, we utilized a monoclonal antibody raised against human fibroblast vimentin that recognizes a nuclear envelope antigen (Callaini and Riparbelli, 1991). This study demonstrated that the antibody strongly stains the nuclear envelope at interphase and throughout the mitotic cycle. However, the exact localization of the antigen on the nuclear envelope and its distribution were still unclear.

This paper re-examines the changing distribution of the nuclear antigen as observed by confocal microscopy, which gives more detailed information than epifluorescence microscopy. Immunoelectron-microscopic techniques also allowed us to detect the spatial localization of this antigen.

Materials and methods

Collection of the embryos

Embryos of *Drosophila melanogaster* (Oregon-R strain) were collected at 24°C on agar plates, dechorionated in a 50% commercial bleach solution, and washed with distilled water. The stage of the nuclear cycle was determined by staining the nuclei with Hoechst 33258.

Immunofluorescence

The dechorionated embryos were fixed according to Warn and Warn (1986), except for a final short fixation with acetone for 5 min. The embryos were then washed in phosphate-buffered saline (PBS) and incubated for 1 h in PBS containing 0.1% bovine serum albumin. After rinsing in PBS the embryos were incubated overnight at 4°C with a monoclonal antibody against vimentin (Sclavo, Siena). This antibody was
made in mouse after immunization with purified vimentin from human cultured fibroblasts. The samples were then washed with PBS and incubated at room temperature in goat anti-mouse fluorescein-conjugated IgG (Cappel, West Chester, PA). For double labeling the embryos were incubated for 4-5 h at room temperature with Rbl88 antiserum, which specifically recognizes an antigen associated with the centrosome of *Drosophila* embryos (Whitfield et al., 1988). These embryos were washed and treated with rhodamine-conjugated goat anti-rabbit IgG (Cappel, West Chester, PA). Nuclei were stained with 1 μg/ml of the DNA-specific dye Hoechst 33258 in PBS. After a final washing in PBS, the embryos were mounted in 90% glycerol containing 2.5% n-propyl gallate (Giloh and Sedat, 1982). Fluorescence observations were carried out with a Leitz Aristoplan microscope equipped with fluorescein, rhodamine and UV filters. Photomicrographs were taken with Kodak Tri-X pan film and developed in Kodak HC 110 developer for 7 min at 20°C.

**Confocal microscopy**

The distribution of the antigen recognized by the anti-vimentin antibody was also described using a Bio-Rad Microsciences (Cambridge, MA) model MRC-500 Confocal Imaging System. This technique provides optical thin sections through the sample. It was configured with a Nikon Optiphot microscope using a ×63 objective.

**Immunoelectron microscopy**

*Drosophila melanogaster* embryos were collected, dechorionated, fixed and devitellinized as described for immunofluorescence. After washing in PBS the embryos were incubated overnight at 4°C in anti-vimentin antibody. The embryos were then washed in PBS and incubated for 1 h at room temperature with anti-mouse IgG conjugated to 10 nm colloidal gold (BioCell, Cardiff). After washing, the samples were fixed in 3% glutaraldehyde in PBS for 30 min, washed again in PBS, stained en bloc with 1% uranyl acetate in water, dehydrated in a graded ethanol series and embedded in Epon. The sections were stained with uranyl acetate and lead citrate and viewed at 60 kV in a Philips EM 400 electron microscope. Control experiments were performed by omitting the incubation with the first antibody.

**Results**

**Preblastoderm embryos**

Whole-mount face views of fixed embryos stained with the anti-vimentin antibody during cycles 1-8, before the somatic nuclei reach the periplasm, show irregular assemblies of anti-vimentin-stained aggregates. Such arrays contain compact and large aggregates of fluorescent-stained material. These aggregates are randomly disposed over the whole surface of the embryo in a 5 μm layer just below the plasma membrane (Fig. 1A). When the somatic nuclei reach the embryo surface during cycle 10 the cortical aggregates are reduced in size and number. Fig. 1D displays a small area of the surface of an embryo at nuclear cycle 10, the first cycle after the nuclei reach the embryo cortex. The antibody strongly stains the nuclear periphery, which appears as a bright envelope-like structure that changes in shape during the mitotic cycle. The cortical aggregates are randomly dispersed among the blastodermic nuclei, but are not localized in cytoplasmic domains like microtubules and microfilaments. As nuclear division progresses the antibody staining is evident around the blastodermic nuclei, whereas the concentration of the antigen in the cytoplasm is less evident and appears as punctate fluorescence (Fig. 1E). The antigen distribution in polar bodies is different from that found in somatic nuclei and no envelope-like structures are observed (Fig. 1B). Double labeling for anti-vimentin and DNA reveals diffuse staining associated with the chromosomes (Fig. 1C). The fluorescence is interrupted in the central region of the polar body where the chromosomes, disposed in a star-like configuration, leave a small gap.

**Syncytial blastoderm**

During interphase the antigen becomes concentrated at high levels around the periphery of the somatic nuclei and is localized in bright rings (Fig. 2A). Suppression of out-of-focus fluorescence and image intensification show that the yolk nuclear periphery is also stained by the antibody (Fig. 2A). The fluorescence encircling the nuclei at prophase is not uniform, but the antibody appears to stain a series of small dots on the nuclear envelope. The antigen is also detected in the surrounding cytoplasm as small fluorescent aggregates. Sagittal optical sections reveal two semiconical structures on the upper side of each somatic nucleus (Fig. 2B). Double fluorescence images with anti-vimentin and Rbl88 antibodies show that these structures coincide with the localization of the centrosomes (Fig. 2C). The antibody continues to stain the polar regions from metaphase to telophase, but the staining becomes diffuse. Fig. 2D shows a double exposure with Rbl88 and anti-vimentin antibodies during anaphase.

The antigen binding pattern changes dramatically during the transition to metaphase. The bright rings are transformed into ovoid structures and the semiconical formations disappear (Fig. 3A). However, strong labeling marks the extremities of the nuclear envelope. In this region the staining is diffuse and we observe a gradient of fluorescence concentration decreasing from the nuclear envelope to the surrounding cytoplasm (Fig. 3A). During anaphase A the staining pattern is similar to that observed during metaphase except for a further elongation of the bright structures and additional fluorescent staining appearing in the equatorial region of the nuclear envelope (Fig. 3B). Anaphase B involves a further elongation of the envelope-like structures, which become elliptical. At this time the antibody labeling is more complex. The extremities of the bright structures are smaller than during anaphase A and the antibody binding pattern has a C-shaped appearance (Fig. 3C). The C-shaped fluorescent extremities become circular during the transition to telophase. At this time we observe small rings, encircling the daughter nuclei, which are connected by interzonal labeling (Fig. 3D). Serial optical sections show that the telophase structures are U-shaped with the nuclei in the upper position. Interzonal labeling is organized in two longitudinal lines in the lower part, whereas fluorescence is diffuse in the upper region (Fig.
Nuclear envelope dynamic in Drosophila embryo

Fig. 1. Localization of the antigen recognized by the monoclonal antibody against human vimentin. (A) Surface view of an embryo during the first nuclear cycle. Antibody labeling appears as irregular fluorescent aggregates. (B,C) Surface view of an embryo during nuclear cycle 2, double labeled with the anti-vimentin antibody and Hoechst 33258. Arrows in B and arrowheads in C indicate the antibody binding pattern and the chromosome disposition in polar bodies. (D) Surface view of an embryo during stage 10. (E) Surface view of an embryo at prophase of stage 11. n, nuclei. Bar, 20 μm.

3D). This staining is the remnant of the anaphase B middle zone staining. As telophase progresses the interzonal fluorescence concentrates in a narrow region between the daughter nuclei (Fig. 3E). At the end of telophase the interzonal labeling reduces and diffuses in the cytoplasm (Fig. 3F). The mitotic stages were determined by simultaneous staining with the DNA-specific dye Hoechst 33258 (not shown).

Discussion

The antigen binding pattern as revealed by epifluorescence and confocal microscopy may be divided into four types of distribution: perinuclear, polar, interzonal and cytoplasmic staining.

The perinuclear staining at interphase is reminiscent of the distribution of the nuclear lamins, which are observed in the form of ring-like structures (Fuchs et al., 1983). However, immunoelectron-microscopic techniques revealed that our antibody recognizes an antigen localized on the outer side of the nuclear envelope. Moreover, the antibody against human fibroblast vimentin strongly stains the nuclear boundary throughout the mitotic cycle (Callaini and Riparbelli, 1991). Instead, the antibodies against the nuclear lamina of Drosophila give a feeble staining during metaphase and anaphase, presumably because the antigens are partly diffuse in the cytoplasm (Fuchs et

Immunoelectron microscopy

To determine the localization of the antigen recognized by the anti-vimentin antibody, we performed immunoelectron-microscopic experiments on Drosophila embryos, which give more detailed structural information. Sagittal views of the nuclear envelope show that gold particles are restricted to the outer side of the nucleus (Fig. 4). Controls using pre-immune serum as first antibody failed to reveal any nuclear envelope staining.
Fig. 2. Scanning confocal microscopic view of *Drosophila* embryos stained with the antibody against human fibroblast vimentin (A,B) and immunofluorescence microscopy of embryos double stained with anti-vimentin antibody and Rb188 serum (C,D). (A) Optical section through a whole mount of an embryo during interphase of nuclear cycle 11 showing the antigen binding pattern around somatic nuclei (sn) and yolk nuclei (yn). (B) Surface view of an embryo during prophase of nuclear cycle 11; arrowheads mark the antigen binding pattern in the centrosomal region. (C) Prophase and (D) anaphase of nuclear cycle 11. Open arrows indicate centrosomes, which are dot-shaped during prophase, and flattened at anaphase. Bars, 10 μm.

The presence of an envelope-like protein, termed otefin, that persists throughout mitosis has recently been described in tissue culture cells and embryos of *Drosophila* (Miller et al., 1985; Harel et al., 1989). The pattern of distribution of otefin is very similar to that observed with anti-vimentin antibody. However, immunoelectron microscopy shows that, in the case of otefin, the gold particles are restricted to the inner membrane of the nuclear envelope, but when the embryos are incubated with anti-vimentin the gold particles are localized on the outer side of the nucleus. The similar immunofluorescence staining, despite the different localization of the antigens, indicates that most of the nuclear envelope is conserved throughout the mitotic cycle, as suggested by the electron-microscopic findings of Stafstrom and Staehelin (1984). When the embryos are observed with a confocal microscope, which permits higher resolution of the fluorescence images, the antigen distribution around the nuclear envelope seems to be discontinuous during mitosis. This is consistent with the observations of Stafstrom and Staehelin (1984), which showed a fenestrated nuclear envelope during the mitotic cycle.

Immunofluorescence observations showed that the antigenic determinant, recognized by the human fibroblast anti-vimentin antibody, is localized at late telophase on the nuclear envelope and in the spindle midzone. Confocal microscopy revealed a distinct flat structure between the re-forming daughter nuclei. Other antigens that show a similar concentration in the spindle midzone have recently been described (Cooke et al., 1987; Kingwell et al., 1987; Sellitto and Kuriyama, 1988; Andreassen et al., 1991; Earnshaw and Cooke, 1991). The gradual diffusion of the midzone staining in the cytoplasm at the end of telophase suggests that the antigen may play a role in nuclear envelope organization during the last phases of mitosis.
This may be confirmed by the observation that at telophase midzone staining is suggested by strong staining of the central region of the nuclear envelope throughout anaphase. Fluorescence staining at anaphase was detected in the region where microtubules overlap antibodies that recognize midzone antigens (see Andreassen et al., 1991). Interzonal labeling at telophase, comparable to that observed with the anti-vimentin antibody, was reported in Drosophila by using an antibody raised against a microtubule-associated protein (Kellogg et al., 1989).

The antigen localization observed in somatic and yolk nuclei was not found in polar bodies. In this case the anti-vimentin antibody did not demonstrate bright rings around the chromosomes, but the fluorescence was diffuse in the nuclear region. This observation may
indicate that polar bodies are unable to form a normal nuclear envelope or, alternatively, that the nuclear envelope antigen has a different localization from that observed in somatic nuclei.

Confocal microscopy shows two semiconical structures localized in the upper region of the blastodermic nuclei during prophase. As mitosis progresses these structures move to the spindle poles and expand, suggesting a relationship with centrosomal behavior. Double-labeling with Rbl88 and anti-vimentin antibodies seems to confirm this hypothesis (Callaini and Riparbelli, 1991; present data). The polar staining observed with anti-vimentin diffuses when the centrosomal material loses its compactness and expands. Electron-microscopic analysis failed to correlate the antigen distribution at the spindle poles with distinct substructures in the nuclear envelope (Stafstrom and Staehelin, 1984; Callaini and Anselmi, 1988). Only a peculiar aggregation of short cisternae near the poles was observed. We can therefore assume that the antigen was also present in these cisternae, but only detailed immunoelectron-microscopic observations can clarify this point. Previous studies have shown an association between centrosomes and nuclear membranes (Bornens, 1977; Nadezhdina et al., 1979; Fais et al., 1984; Katsuma et al., 1987). If a linkage between centrosomes and the nuclear envelope exists, it seems to be a feeble interaction, since there are several cases in which the centrosomes detach from the spindle poles. *Drosophila* embryos microinjected with anti-tubulin antibodies (Warn et al., 1987), lethal maternal mutant gnu (Freeman et al., 1986), aphidicolin-treated embryos (Raff and Glover, 1988) and UV-irradiated embryos (Yasuda et al., 1991) have shown isolated centrosomes. To determine whether the cross-reacting *Drosophila* antigen remains associated with centrosomes once they dissociate from nuclei, we followed the behavior of the yolk nuclei. In normally developing embryos the yolk centrosomes continue their replication although the yolk nuclei cease to divide. Moreover, these centrosomes separate from the yolk nuclei and move into the cytoplasm, thus providing an excellent natural system for the study of the uncoupling of the nuclear and centrosomal cycles (Callaini et al., 1990; Callaini and Dallai, 1991). In this condition polar staining was not observed (unpublished data), suggesting that the labeling is closely related to the position of the centrosome on the nuclear envelope.

Since from fertilization to cellular blastoderm formation the surface area of the nucleus increases several times, a large amount of karyoskeletal proteins is required for the correct assembly of nuclei in rapidly developing early embryos. The presence of many fluorescent aggregates in the cortical cytoplasm of early embryos before the beginning of nuclear division suggests that the antigen recognized by the anti-vimentin antibody might be maternally supplied to the embryo. This has already been observed for *Drosophila* lamins and a group of nuclear envelope proteins, which are partly dispersed in the cytoplasm during mitosis (Frash et al., 1988). The fluorescent aggregates undergo rapid disassembly after cycle 10, when the first somatic nuclei reach the cortex, and only punctate fluorescence is visible during the last syncytial mitoses. This indicates a direct relationship between nuclear density and quantity of fluorescent aggregates in the peripheral cytoplasm and suggests that this antigen shifts into the nuclear compartment. Other proteins such as actin (Warn et al., 1984; Karr and Alberts, 1986; Hatanaka and Okada, 1991), tubulin (Karr and Alberts, 1986; Warn and Warn, 1986; Kellog et al., 1988; Warn et al., 1990), myosin (Warn et al., 1979, 1980; Lutz and Kiehart, 1987; Young et al., 1991), spectrin (Pesacreta et al., 1989), actin-binding proteins (Miller et al., 1989) and intermediate-like filaments of the vimentin type (Walter and Alberts, 1984; Biessman and Walter, 1989) have been observed in the cortex of the preblastoderm embryo. These proteins, which are observed in cytoplasmic domains, undergo drastic reorganization after nuclear migration to the periphery.

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