Tyrosine phosphorylation accompanying the cellularization of the syncytial blastoderm of *Drosophila*

(embryonic development/epithelium formation/immunofluorescence microscopy)

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ABSTRACT At an early stage in embryogenesis, the *Drosophila* blastoderm is a syncytium in which ≈ 6000 nuclei align under the plasma membrane. During the interphase of nuclear cycle 14, a wave of membrane formation descends from the blastoderm surface to enclose each nucleus in an intact cell membrane. We show by immunofluorescence microscopy that the membrane-formation process is closely accompanied in space and time by a wave of tyrosine phosphorylation, suggesting that one or more tyrosine kinases and phosphatases are active in the process.

During the early stages of development of the *Drosophila* embryo, a syncytium is formed in which ≈ 6000 somatic nuclei become organized in the peripheral cytoplasm underneath the plasma membrane of the blastoderm. During the following interphase of nuclear cycle 14, a synchronized wave of membrane formation descends from the plasma membrane of the blastoderm into the periplasm and within ≈ 1 hr surrounds each nucleus in its own plasma membrane. This process of membrane encirclements of the nuclei generates the epithelial cell layer of the cellular blastoderm (for reviews, see refs. 1 and 2).

We have recently (3) provided electron microscopic evidence that the sources of these membranes are intraembryonic membrane-encircled vesicles, of diameters from 0.05 μm to 0.5 μm , which are present in the periplasm and become redistributed during cellularization. Fusions of these vesicles with one another and with the forming double membranes apparently generate the cellular membranes.

To explore further the molecular mechanisms of cell-membrane formation, we investigated whether the phosphorylation of protein tyrosine residues occurs during the process and whether it correlates with any of the morphological events of cellularization. By genetic analyses, several tyrosine kinases have been shown to be important in various stages of *Drosophila* embryonic development (4–6), and in other systems, tyrosine kinases are active in embryonic epithelial-cell-junction formation *in vivo* (7). To examine this question, we prepared semi-thin frozen sections of appropriately fixed embryos during the first several hours of development and immunofluorescently labeled the sections by using antibodies specifically directed to phosphotyrosine (*P*-Tyr) residues.

Under the conditions of our experiments, *P*-Tyr-modified proteins were not detected (apart from the yolk granules) in the apical periplasm until the beginning of the second hour of development. Thereafter, the distribution of such *P*-Tyr-modified proteins at the light-microscopic level of resolution changed regularly with the cellularization stage and paralleled distinctive features of the cellularization process previously seen in the electron microscope. These findings suggest an important role for this enzymic modification in regulating the process of cell-membrane formation.

MATERIALS AND METHODS

***Drosophila melanogaster* Embryos and Specimen Preparation.** *Drosophila* eggs were provided through the kindness of James Kadonaga and were collected, sampled at various times, and dechorionated, all as described (3). After dechorionation, the embryos were immobilized on double-sided tape and covered by a drop of fixative (4% paraformaldehyde/0.1 M phosphate, pH 7.4, containing 0.1 M *O*-vanadate to inhibit tyrosine phosphatase activity). About 10 μl of fixative was then gently microinjected (3) through a fine puncture in the vitelline membrane at the fluid-filled pockets of the embryos. After another 5 hr in the fixative, the now rigid vitelline membrane was removed by gentle microneedle manipulation. The devitellinized embryos were then washed in the phosphate buffer and immersed in 2.3 M sucrose, mounted on pins, rapidly frozen in liquid N_2 , and cryosectioned into 2- to 3- μm -thick sections with a Reichert model OMV-4/FC cryoultramicrotome at -60°C (8). The sections were cut randomly in different projections, although only longitudinal ones are shown in Figs. 1 and 2. This technique allowed inspection of the immunolabeling of entire zygotes and blastoderms and insured that the features described below were consistently observed. Within the time interval after nuclear division 14 (2.5–3.5 hr), more precise ordering of the developmental sequence was based on morphological criteria in the Nomarski images, such as the state of membrane furrow formation and the shapes and positions of the nuclei (3, 9).

Immunofluorescent Labeling and Microscopy. The frozen sections were transferred onto polylysine-coated glass slides, thawed by washing in the phosphate buffer, treated with 3% normal goat serum to block nonspecific binding, and incubated with affinity-purified rabbit polyclonal antibodies (100 $\mu\text{g}/\text{ml}$) directed to *P*-Tyr (7) for 30 min at room temperature. This procedure was followed by washing and incubation with rhodamine-conjugated goat anti-rabbit IgG antibodies for 20 min; then the slides were washed and stained with Hoechst 33258 for 2 min to stain the nuclei. Finally the slides were washed, mounted in 90% (vol/vol) glycerol/10% (vol/vol) 0.1 M Tris buffer, pH 8.0/0.1% *p*-phenylenediamine and examined with a Zeiss Photoscope III epifluorescence instrument equipped with Nomarski optics. The specificity of anti-*P*-Tyr immunolabeling was tested by experiments in which the anti-*P*-Tyr antibody solution contained, in addition, either 10 mM *O*-*P*-Tyr or *O*-phosphoserine.

RESULTS

Tyrosine phosphorylation was detected by immunofluorescence in the yolk granules at all stages from the zygote through cellularization (Figs. 1 *b* and *d* and 2*d*). All immunolabeling

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Abbreviation: *P*-Tyr, phosphotyrosine.

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FIG. 1. Semi-thin frozen sections of the *Drosophila* blastoderm during the first two hours of development before cellularization, indirectly immunolabeled with anti-*P*-Tyr antibodies (*b*, *d*, and *f*); each field is accompanied by its Nomarski differential interference image and also shows the nuclei labeled with Hoechst 33258 stain (*a*, *c*, and *e*, respectively). Most yolk granules (Y), of various sizes and of random distribution, were immunolabeled for *P*-Tyr (*b* and *d*). There was no detectable variation in the pattern or amount of labeling of the yolk granules during the first four hours of development. (*a* and *b*) By the end of the first hour (*a*) some of the nuclei (N, arrowheads) have moved to the periphery of the blastoderm, and others (*n*) are still in the interior. Neither the periplasm (P in *b*) nor the plasma membrane (arrowheads in *b*) shows *P*-Tyr immunolabeling. (*c* and *d*) At a slightly later time (≈ 1 – 1.5 hr) the periplasm showed discrete punctate *P*-Tyr immunolabeling close to the blastoderm surface (arrows in *d*), while the plasma membrane (arrowheads) was not labeled. (*e* and *f*) In the next half hour, the punctate *P*-Tyr immunolabeling persisted (arrows in *f*) but was now closely opposed to the plasma membrane, which itself appeared labeled. (Bar = $20\ \mu\text{m}$ for *a*–*f*).

observed was specific for *P*-Tyr residues because it was eliminated when labeling was done in the presence of excess free *P*-Tyr but was unaffected by excess free phosphoserine (data not shown). Although this yolk granule labeling is of interest, the unchanging character of the labeling suggested that it was not germane to the process of cellularization, and it was therefore not further explored in this study.

The only other specific immunolabeling for *P*-Tyr was detected in the periplasmic space and associated with the forming cell membranes in a time-dependent way. Such immunolabeling was absent from the zygote (data not shown) and up to 1 hr of development of the syncytial blastoderm (Fig. 1*b*). The first detectable labeling appeared at the beginning of the second hour (Fig. 1*d*), in a finely punctate distribution near the blastoderm surface. At this stage, the plasma membrane of the blastoderm was unlabeled. During the following half hour (i.e.,

1.5- to 2-hr development time), this punctate labeling moved closer to the surface, and the membrane itself now appeared labeled (Fig. 1*f*). With the beginning of cellularization (≈ 2.5 hr), at a stage when the peripherally located nuclei were still largely spherical (Fig. 2*a*) and the surface of the blastoderm did not yet show much membrane furrowing (corresponding to phase I of ref. 3), *P*-Tyr labeling was observed in linear arrays between the nuclei (Fig. 2*b*), extending from the now unlabeled plasma membrane of the blastoderm ≈ 7 – $9\ \mu\text{m}$ into the periplasm.

With further development (≈ 3 hr), the *P*-Tyr labeling (Fig. 2*d*) extended the entire lengths ($\approx 20\ \mu\text{m}$) of what were clearly the newly forming double membranes, including those few regions that had already completely enveloped a nucleus at this time (Fig. 2*d*, lower right arrowhead). The regions of the plasma membrane at the blastoderm surface, however, were

FIG. 2. Semi-thin frozen sections as in Fig. 1 but taken at later cellularization stages. (a and b) At the beginning of the process (≈ 2.5 hr of development, corresponding to phase I of ref. 3), the now peripherally localized nuclei (N) were still largely spherical, and the blastoderm surface was unfurrowed (a). *P*-Tyr immunolabeling was confined to short linear segments (arrows in b) extending from the blastoderm surface between successive nuclei. The blastoderm surface is unlabeled. P, perioplasm. (c and d) At a later stage (≈ 3 hr, corresponding to phase III of ref. 3), the nuclei were elongated, and the blastoderm surface was furrowed (c). *P*-Tyr immunolabeling extended the entire lateral lengths of the forming membranes (arrows in d) and was also present on the basal surface (lower right arrowhead in d), where membrane encirclement had apparently just occurred. Note the striated *P*-Tyr immunolabeling in a cluster of the forming cells (upper left arrowhead in d), terminating in fluorescent buttons at the double membranes. The blastoderm surface is unlabeled. (e and f) At a still later stage (≈ 3.5 hr), when cellularization was complete, only the basal surfaces of the cellular membranes (arrowheads in f) showed *P*-Tyr immunolabeling, which is circumferential (lower arrow in f). (Bar = $20 \mu\text{m}$ for a-f.)

not labeled. In several of the adjacent cellularizing units, cross-striations of *P*-Tyr labeling, regularly spaced about $2 \mu\text{m}$ apart, were observed (Fig. 2d, upper left arrowhead). Probably these units were fortuitously sectioned through one transverse plane, whereas the other units that did not exhibit the cross-striations were sectioned through other transverse planes in the 2- to $3\text{-}\mu\text{m}$ sections. We believe that this striated labeling was closely associated with the inner surfaces of the forming double plasma membranes because where the striations ended at the membranes, a small button of fluorescence was observed (Fig. 2d), suggesting that a junction was formed between the double membranes at such sites.

Upon still further development (≈ 3.5 hr), *P*-Tyr labeling was detectable only at the basal surfaces (Fig. 2f) of the now fully formed cells. Where these cells were sectioned obliquely (Fig. 2f, bottom arrow), the basal surface labeling was seen to be

circumferential. This pattern of basal *P*-Tyr labeling did not change significantly over the ≈ 10 -hr period of development that we examined (data not shown).

DISCUSSION

Our previous electron microscopic studies (3) led us to propose that the process of membrane formation during cellularization of the syncytial blastoderm of *Drosophila* mainly involves an hour-long concerted sequence of extensive redistributions and fusions of intraembryonic small vesicles. In this paper, we find that the changing distribution of high densities of protein tyrosine phosphorylation correlates strongly with this sequence of structural events in the membrane-formation process. This result suggests that one or more tyrosine kinase activities play important roles in the process.

Neither the zygote, nor the syncytial blastoderm during the first hour of development, showed detectable levels of *P*-Tyr immunofluorescent labeling by our procedures, apart from the yolk granules. In particular, the plasma membrane and periplasmic space (Fig. 1*b*) of the zygote and first-hour blastoderm exhibited no significant labeling. By the beginning of the second hour of development, however, discrete punctate labeling near the plasma membrane was seen (Fig. 1*d* and *f*). We believe that this labeling is associated with the multitude of small vesicles that we previously observed to occupy a substantial fraction of the apical periplasm at this stage of embryonic development (compare figure 1*A* of ref. 3). Our morphological studies showed that the subsequent onset of cellularization was signaled by an alignment of these small vesicles between the successive spherical nuclei, extending from the blastoderm surface about halfway down the length of the nuclei (compare figures 1*B* and 2*B* of ref. 3); this stage was designated phase I of the membrane-formation process (3). The *P*-Tyr labeling pattern in Fig. 2*b* in the present paper corresponds closely in time and position to the lined-up vesicles in phase I. These vesicles are about to undergo concerted fusions with one another and with the plasma membrane of the blastoderm (designated phase II in ref. 3) to generate a growing double membrane. Note that in contrast to the previous stage (Fig. 1*f*), the unfurrowed plasma membrane at the blastoderm surface and the apical periplasm were no longer immunolabeled in phase I (Fig. 2*b*).

In a later period, designated phase III (3), the double membranes were further extended beyond the now-elongated nuclei and eventually encircled them. This phase corresponds structurally to the *P*-Tyr immunolabeling pattern in Fig. 2*d*. Although the surface of the blastoderm was unlabeled, the entire lateral regions of the double membranes, as well as the newly formed basal region (Fig. 2*d*, lower right arrowhead), were immunolabeled. At a still later stage, when cellularization was mostly completed (Fig. 2*f*), the lateral regions of the membranes were no longer immunolabeled, and only the basal membrane regions exhibited labeling.

Thus, a wave of protein tyrosine phosphorylation appears to be closely associated with membrane formation during cellularization, starting before cellularization in discrete bodies that are probably vesicles at or near the plasma membrane at the blastoderm surface, then concentrating at the lateral regions of the forming double membranes, and finally being confined to the basal regions of the membranes at the end of the process. This wave is presumably the consequence of the sequential activations of one or more membrane-associated protein tyrosine kinases and phosphatases; these, as well as their protein substrates, await identification. In this connection, it is important to realize that the presence of a particular enzyme

associated with the forming membranes does not necessarily implicate it in the activities observed. Thus, the integral membrane protein tyrosine kinase that is the product of the torso gene appears to be located on all of the forming membranes (4), but its activity during early embryogenesis is apparently confined to the blastoderm poles (10). This result implies that the required ligand for the torso kinase may only be present near the poles.

We have as yet no direct evidence about the function or functions served by this wave of tyrosine phosphorylation. In other instances of possible relevance, however, tyrosine phosphorylation has been implicated in vesicle-fusion events (compare refs. 11 and 12), such as we have proposed occur during membrane formation (3); in transmembrane signaling (see ref. 13); and in the formation of cell-substrate and cell-cell junctions (see ref. 14). The striated *P*-Tyr immunolabeling pattern seen in Fig. 2*d* (left upper arrowhead), forming small fluorescent buttons at what appear to be intermembrane junctions, may reflect one role for tyrosine phosphorylation in the formation of junctions between the two juxtaposed members of a double membrane.

The present observations, therefore, open up many avenues for further investigation.

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