

Nuclear envelope breakdown in mammalian cells involves stepwise lamina disassembly and microtubule-driven deformation of the nuclear membrane

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SUMMARY

We have studied nuclear envelope disassembly in mammalian cells by morphological methods. The first signs of nuclear lamina depolymerization become evident in early prophase as A-type lamins start dissociating from the nuclear lamina and diffuse into the nucleoplasm. While B-type lamins are still associated with the inner nuclear membrane, two symmetrical indentations develop on antidiapetric sites of the nuclear envelope. These indentations accommodate the sister centrosomes and associated astral microtubules. At mid- to late prophase, elongating microtubules apparently push on the nuclear surface and eventually penetrate the nucleus. At this point the nuclear envelope becomes freely permeable to large ligands, as indicated by experiments with digitonin-treated cells and by the massive release of solubilized A-type lamins into the cytoplasm. At the prophase/prometaphase transition, the

B-type lamina is fragmented, but 'islands' of lamin B polymer can still be discerned on the tips of congressing chromosomes. Finally, at metaphase, the lamin B polymer breaks down into small pieces, which tend to concentrate in the area of the mitotic spindle. Nuclear envelope breakdown is not prevented when the microtubules are depolymerized by nocodazole; however, the mode of nuclear lamina fragmentation in the absence of microtubules is markedly different from the normal one and involves multiple ruffles and gaps, which develop rapidly along the entire surface of the nuclear envelope. These data suggest that nuclear envelope disassembly is a stepwise process in which the microtubules play an important part.

Key words: Nuclear envelope, Nuclear lamina, Microtubule, Mitosis

INTRODUCTION

The cell nucleus is confined by a tight, membranous envelope. The nuclear envelope comprises the inner and outer nuclear membranes, which join at the region of the nuclear pores. The outer nuclear membrane represents an extension of the rough endoplasmic reticulum (RER), whereas the inner nuclear membrane and the pore membrane have a unique composition and contain specific resident proteins (for reviews see Gerace and Burke, 1988; Nigg, 1989; Goldberg and Allen, 1995; Wilson and Wiese, 1996).

Underlying the nuclear envelope is a meshwork of intermediate filaments, the nuclear lamina (Aebi et al., 1986). The lamina is a polymer of A- and B-type lamins tethered to the inner nuclear membrane through several integral membrane proteins (Nigg, 1992; Gerace and Foisner, 1994; Georgatos et al., 1994). These lamin-binding integral membrane proteins include the lamin B receptor (LBR; Worman et al., 1988), the lamina-associated polypeptides (LAP1A, LAP1B, LAP1C and LAP2; Senior and Gerace, 1988; Foisner and Gerace, 1993) and a recently identified protein termed p18 (Simos et al., 1996).

In some unicellular eukaryotes, the nuclear envelope retains its physical continuity during mitosis ('closed' mitosis). However, in higher eukaryotic cells this membranous organelle

is completely disassembled at the onset of cell division ('open' mitosis). Nuclear envelope breakdown involves two temporally and mechanistically distinct processes: nuclear lamina depolymerization and fragmentation of the nuclear membrane (Newport and Spann, 1987; Peter et al., 1990). There is general consensus that lamina depolymerization is the consequence of p34/cdc2-mediated phosphorylation (reviewed by Nigg, 1992), but the intermediates of lamina disassembly have not yet been characterized.

Although several interesting ideas have been proposed (Warren, 1993), the exact mechanism of nuclear membrane fragmentation remains elusive. For instance, it has not been resolved whether all the connections between the lamina and the inner nuclear membrane are severed prior to membrane breakdown, and whether the outer nuclear membrane fragments independently from, or together with, the inner membrane. Pioneering work (Robbins and Gonatas, 1964; Murray et al., 1965; Bajer and Bajer, 1969) has suggested that the nuclear envelope of mitotic cells undergoes a series of structural changes that commence at early prophase. However, these early studies did not resolve the fine details of nuclear envelope disassembly, as they were performed before the advent of reliable immunohistochemical methods and the discovery of the nuclear lamins.

More recent studies involving *Drosophila* early embryos

have shed some light into the mitotic reorganization of the nuclear envelope. Nuclear disassembly in *Drosophila* early embryos seems to follow a pattern that is intermediate between traditional 'open' and 'closed' mitosis. Furthermore, nuclear lamina depolymerization seems to be a late event, which occurs at about the time the spindle has attained its final metaphase configuration (Strafstrom and Staehelin, 1984; Harel et al., 1989; Paddy et al., 1996). Analogous immunocytochemical studies in mammalian cells have been limited by the scarcity of prophase and prometaphase figures in unsynchronized cultures.

Searching for a suitable mammalian system that could be used for studying nuclear envelope disassembly under in vivo conditions, we came across the Ishikawa cells, a human endometrial adenocarcinoma cell line (Nishida et al., 1985), which is naturally enriched in early mitotic figures. Employing this model and using specific markers we have been able to examine nuclear envelope disassembly in situ. Results described below reveal that nuclear envelope breakdown is a stepwise process that involves sequential lamina disassembly and microtubule-driven deformation of the nuclear membrane. These observations have been extended by performing similar studies on a widely used rodent line, normal rat kidney (NRK) cells.

MATERIALS AND METHODS

Cell maintenance and treatment

Human endometrial adenocarcinoma (Ishikawa) cells were cultured in MEM, while normal rat kidney cells (NRK) were maintained in DMEM. Culture media contained 10% FBS and antibiotics. To depolymerize the microtubules, the cells were chilled on ice for 30 minutes and then bathed in culture medium containing 33 μ M nocodazole for 30 minutes at 37°C.

Antibodies

Microtubules were stained using two commercially available monoclonal antibodies recognizing α - and β -tubulin and a rabbit polyclonal antibody developed against α -tubulin (courtesy of E. Karsenti, EMBL, Heidelberg, FRG). Vimentin filaments were stained with the monoclonal antibody 7A3 (Papamarcaki et al., 1991), and keratin filaments were decorated by a commercially available monoclonal antibody. The lamins were specifically stained using the anti-peptide antibody No. 16 (developed against the tail domain of mouse lamin B1), the anti-peptide antibodies No. 14 and No. 15 (raised against the head region of human lamins A/C), the anti-peptide antibody No. 63 (raised against the tail domain of lamin A) and the monoclonal anti-lamin A/C antibody XB-10 (a generous gift from B. Burke). All anti-lamin antibodies have been characterized by western blotting and indirect immunofluorescence microscopy (Simos and Georgatos, 1992; Meier and Georgatos, 1994; Maison et al., 1995; Pyrpassopoulou et al., 1996; Powell and Burke, 1990). The characterization of the anti-LAP1 antibodies will be reported elsewhere (Maison et al., 1997).

Indirect immunofluorescence and electron microscopy

Indirect immunofluorescence microscopy was performed as described previously (Meier and Georgatos, 1994; Maison et al., 1995). Under standard conditions, the cells were fixed with 4% formaldehyde in PBS for 10 minutes at room temperature. In other cases, fixation was accomplished using a mixture of methanol/acetone (1:9) for 2 minutes at room temperature or at -20°C. The cells were permeabilized with 0.5% Triton X-100 or 0.002% digitonin, depending on the experimental circumstances. Following permeabilization, the specimens

were incubated in PBS, 0.5% Triton X-100 and 1% fish skin gelatin (except in cases where permeabilization involved digitonin) and stained with primary and secondary antibodies. Several secondary antibodies were used, including goat anti-rabbit, rabbit anti-mouse and donkey anti-rabbit or anti-mouse immunoglobulins conjugated to FITC, Texas Red and rhodamine. The chromosomes were decorated by the DNA-binding dye DAPI. Specimens were visualized in a Zeiss Axioscope microscope or a confocal laser scanning microscope, constructed and maintained at EMBL (Heidelberg, FRG).

For electron microscopy, cells were fixed with 1% glutaraldehyde and 1.5% tannic acid, osmicated, Epon embedded and stained with heavy metals. Specimens were visualized in a Philips 400 electron microscope operated at 80 kV.

RESULTS

Disassembly patterns of A- and B-type lamins

Staining of Ishikawa cells with specific antibodies revealed that B-type lamins remain well localized at early phases of mitosis. However, concomitant with chromosome condensation, the nuclear lamina meshwork appeared to undergo a series of structural changes. At early prophase, the lamina exhibited a focal indentation (Fig. 1a/a'), or a deep invagination (Fig. 1b/b'). The lips of this invagination were lined by densely packed chromatin and were often in touch with one another, creating the impression of a longitudinal fold (Fig. 1b/b' and Fig. 4b shown below). At mid-prophase, a second indentation emerged antidiagonally to the first one and the nucleus acquired a clepsydra (water clock)-like form (Fig. 1c/c' and Fig. 3c' shown below). Finally, at late prophase/prometaphase, the B-type lamina became fragmented (but not completely dissolved) and one could distinguish fragments of the B-type polymer covering congressing chromosomes (Fig. 1d/d').

A-type lamins appeared to follow a different disassembly stereotype, dissociating from the nuclear envelope already at early prophase. The depolymerized material released from the nuclear envelope diffused first into the nucleoplasm and occupied the void between condensed chromosomes (Fig. 2b/b'), suggesting that the nuclear envelope was still intact (see also below). Subsequently, A-type lamins broke through the nuclear envelope and appeared in the cytoplasm (Fig. 2c/c'). Thus, by late prophase only a thin layer of A-type lamins remained on the surface of chromosomes, while the bulk of these proteins was diffusely distributed throughout the cytoplasm (Fig. 2d/d').

The focal deformation of the B-type lamina does not involve blebbing and dissociation from the inner nuclear membrane

To find out whether the deformation of the nuclear envelope involved local dissociation of the B-type lamina from the inner nuclear membrane (blebbing), we performed double immunolabelling experiments using anti-lamin B antibodies and antibodies against the lamina-associated polypeptide-1C (LAP1C), an integral protein of the inner nuclear membrane that binds tightly to the nuclear lamins (Senior and Gerace, 1988; Foisner and Gerace, 1993; Maison et al., 1997). These experiments were done in NRK cells, which behaved exactly as Ishikawa cells in all aspects of nuclear envelope disassembly, because the anti-LAP1 antibodies were rat-specific. As shown in Fig. 3a/a'-d/d', the distribution patterns of LAP1C and lamin B were

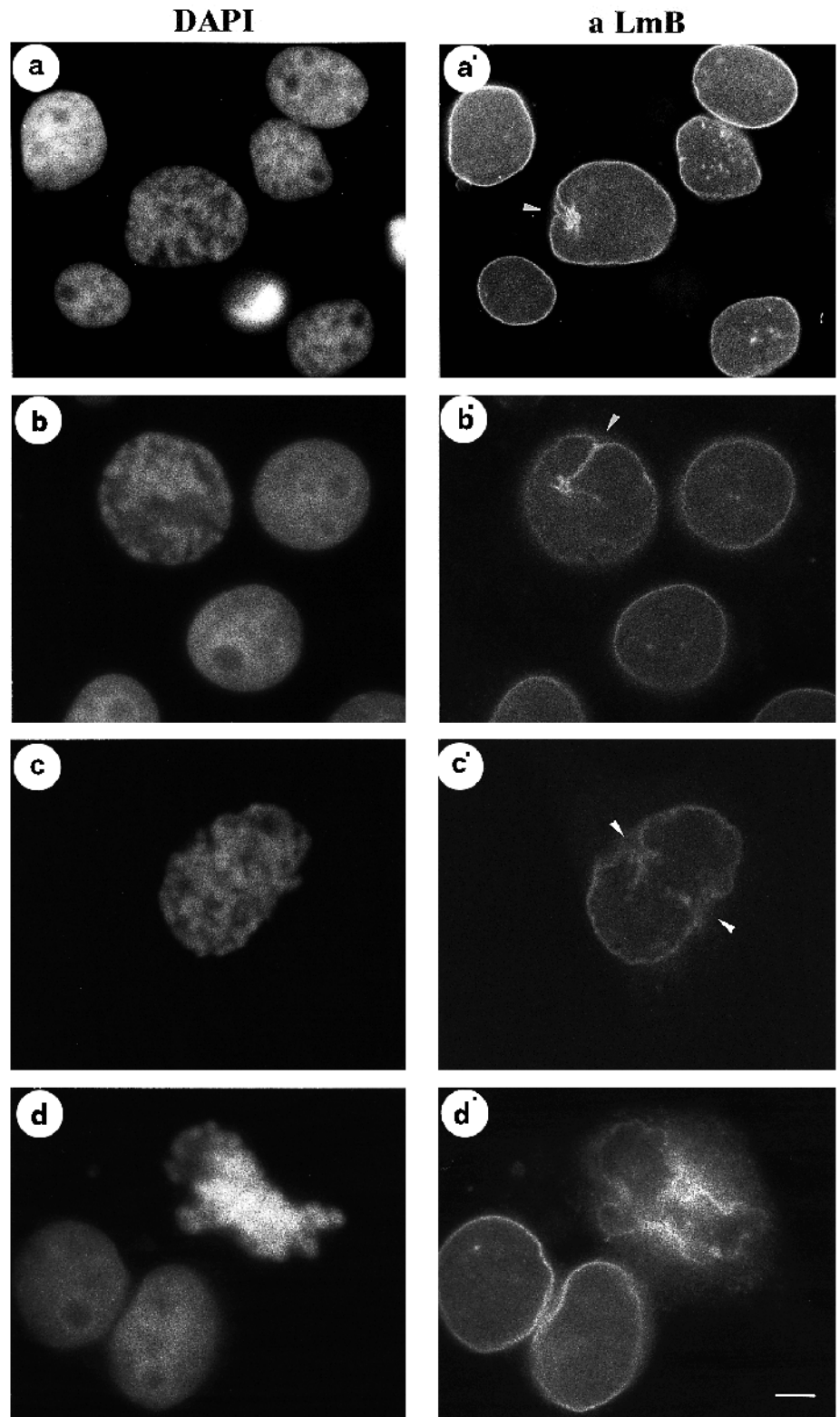


Fig. 1. Disassembly of B-type lamins in the early stages of mitosis. Unsynchronized Ishikawa cultures were stained with DAPI and anti-lamin B antibodies (a LmB) and examined by conventional indirect immunofluorescence microscopy. The pairs a/a'-c/c' show successive stages of prophase, while d/d' depicts a cell in late prophase/prometaphase. Note the indentations of the B-type lamina (arrowheads) and the partial fragmentation of the polymer at prometaphase. Bar, 3 μ m.

exactly superimposable over the 'hills' and 'valleys' of the nuclear surface that could be distinguished during prophase. Hence, it is unlikely that the focal deformation of the nuclear envelope is due to dissociation of the B-type lamina from the inner nuclear membrane.

The deformation of the prophase nuclear envelope is caused by growing microtubules

Examination of Ishikawa cells by electron microscopy showed that the indentations of the nuclear envelope were invaded by bundles of cytoskeletal elements containing

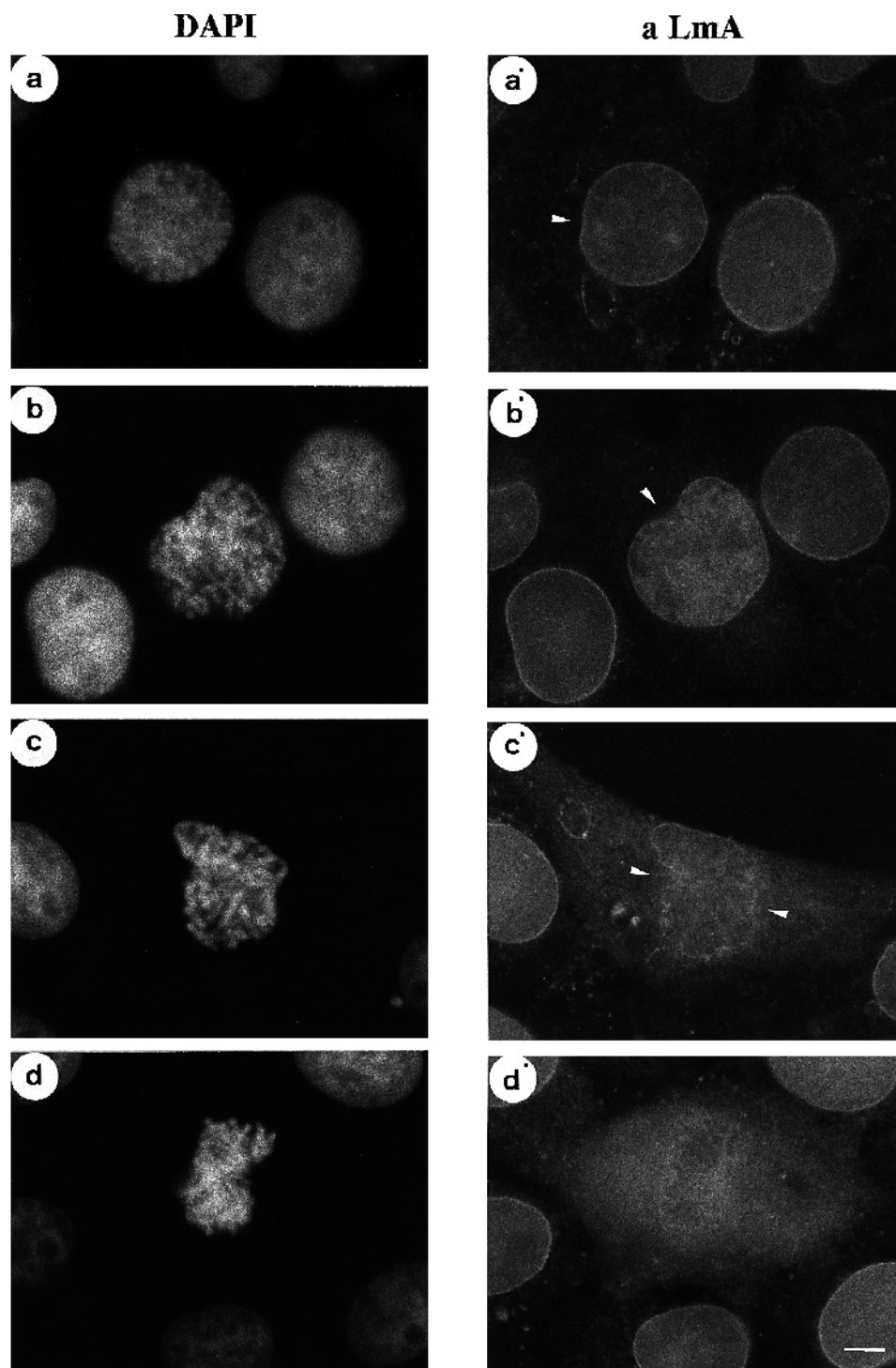


Fig. 2. Disassembly of A-type lamins in the early phases of mitosis. Unsynchronized Ishikawa cultures were stained with DAPI and anti-lamin A antibodies (a LmA) and examined by conventional indirect immunofluorescence microscopy. The pairs a/a'-c/c' show successive stages of prophase, while d/d' depicts a cell in late prophase/prometaphase. (a/a') A cell with a subtle indentation (arrowhead) in very early prophase in which lamin A is still well localized. At a subsequent step (b/b'), as the indentation deepens (arrowhead), lamin A dissociates from the nuclear envelope and fills the nucleoplasm. Finally, lamin A material leaks to the cytoplasm when the cells reach the state of the 'clepsydra' (arrowheads) (c/c'). Bar, 3 μ m.

microtubules and thinner fibers that resembled intermediate filaments (Fig. 4a-d). Consistent with this observation, double labelling with anti-tubulin and anti-lamin B antibodies and examination of specimens by confocal microscopy showed that the two antidiаметrical indentations accommodated precisely the sister centrosomes and the associated microtubule asters (Fig. 4e,f). Although vimentin and keratin intermediate filaments could also be detected in the neighborhood of the prophase nucleus, these structures were not 'focused' at the two indentations as were the microtubules (data not

shown). Consistent with Fig. 2d/d', further analysis showed that at later phases of nuclear envelope disassembly membranous structures, which probably represent fragments of the disassembled nuclear envelope, aligned along kinetochore microtubules or attached to the tips of the chromosomes (Fig. 4g). From the sum of these observations it would appear that spindle growth and nuclear envelope disassembly proceed in a highly coordinated fashion.

Supporting this point, the one-to-one correlation between microtubule asters and nuclear indentations was particularly

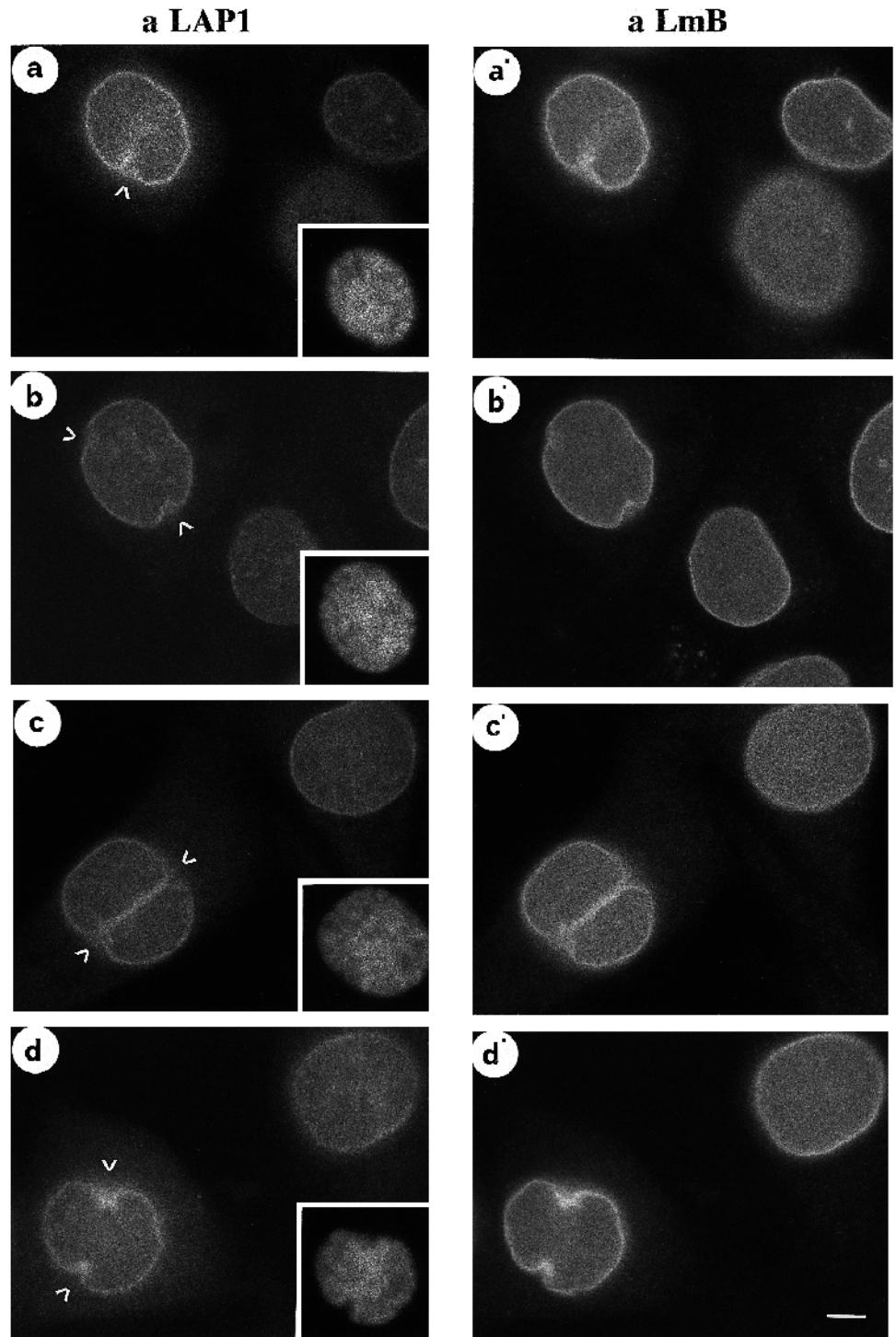


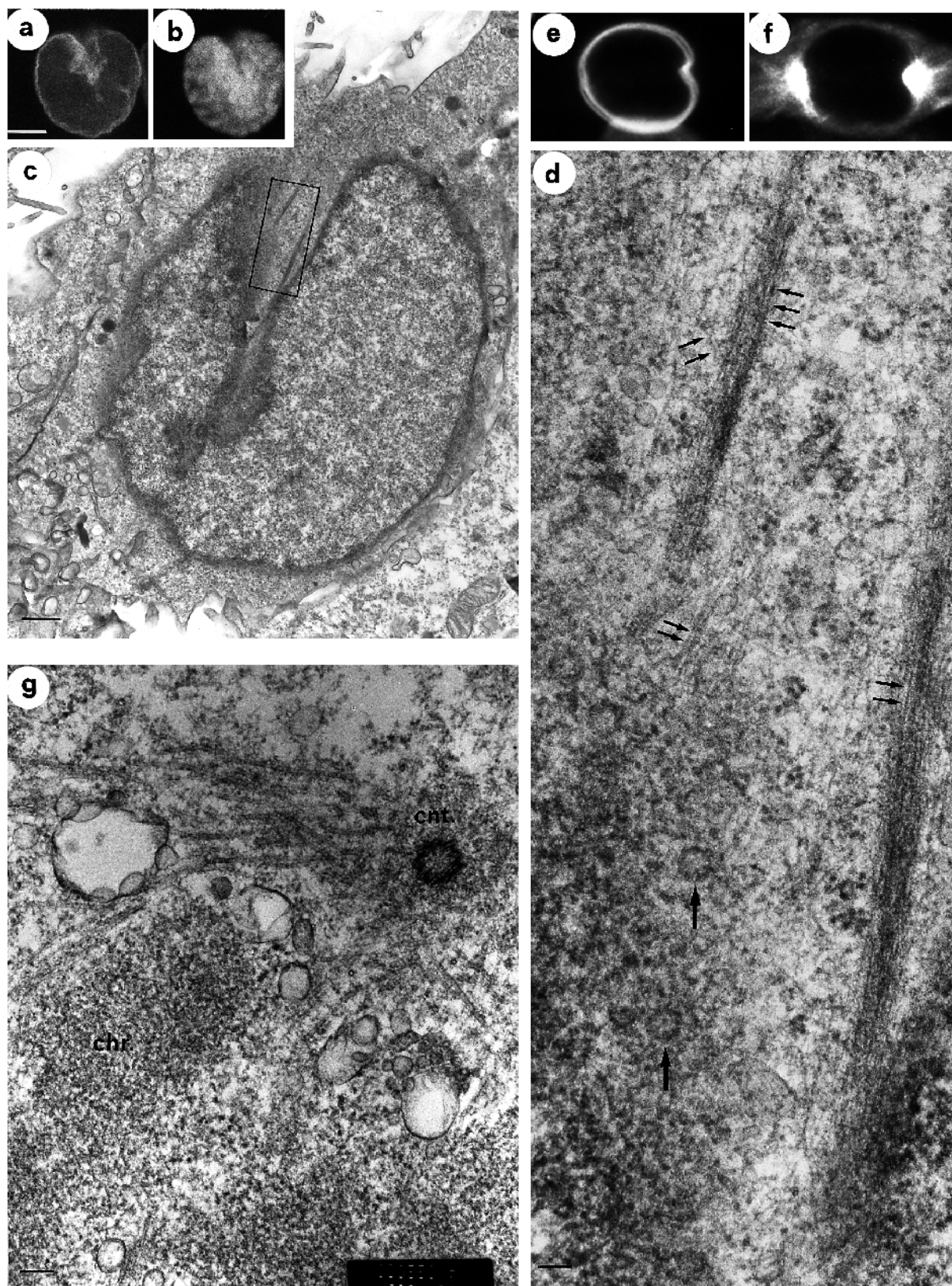
Fig. 3. Co-localization of lamin B and LAP1C during prophase.

Unsynchronized NRK cultures were stained with anti-LAP1A/C (a LAP1) and anti-lamin B antibodies (a LmB) and examined by conventional indirect immunofluorescence microscopy. The pairs a/a'-d/d' show successive stages of prophase. Notice the exact co-localization of the two antigens in all 'hills' and 'valleys' that develop on the nuclear surface (arrowheads). Bar, 3 μ m.

evident when we examined rare mitotic figures possessing more than the normal number of asters and stained doubly with anti-lamin and anti-tubulin antibodies. As shown in Fig. 5, opposite each aster there was a focal depression of the nuclear lamina, which created the impression of a multilobulated nucleus. In situ data (Fig. 6a/a',b/b') demonstrated further that, during prophase, microtubules growing from the two sister centrosomes extended into and filled the deep grooves which develop on the surface of the nucleus. At a subsequent step, prometaphase, the microtubules invaded the nuclear interior,

while fragments of the lamin B polymer remained associated with the tips of the chromosomes (Fig. 6c/c'). By metaphase, most of the lamin B polymer had disassembled, whereas the mitotic spindle had fully assembled. At that stage, a fraction of B-type lamins were detected in close proximity to the mitotic spindle, whereas another fraction was localized at more peripheral sites of the cytoplasm (Fig. 6d/d').

To find out whether microtubules growing from the centrosomes were responsible for the characteristic deformation of the nuclear envelope during prophase, we examined cells



that had been treated with microtubule depolymerizing agents. To effect microtubule depolymerization *in vivo*, we treated Ishikawa cells with 33 μ M nocodazole for 30 minutes. As seen in Fig. 7a/a', early and mid-prophase cells that had

their microtubule system destroyed did not possess the characteristic nuclear indentations. Instead, their laminae were uniformly 'ruffled'. At a slightly more advanced stage of prophase, the B-type lamina appeared to be 'pulverized' (Fig.

Fig. 4. Localization of microtubule asters at the indentations that develop during prophase. Mitotic Ishikawa cells were examined by indirect immunofluorescence (a,b,e,f) and electron (c,d,g) microscopy. (a,b) A prophase cell decorated with anti-lamin B antibodies and DAPI, respectively; (c) a morphologically similar figure embedded in Epon, thin-sectioned and visualized at the electron microscope; (d) enlargement of the area shown in c; (e,f) a prophase cell doubly stained with anti-lamin B and anti-tubulin antibodies, respectively; (g) thin section of a prometaphase cell depicting the area of the developing spindle. Small arrows in d indicate microtubules, which organize as bundles and invade the deep indentation of the nucleus. Large arrows point to nuclear pore complexes; chr. indicates the chromosomes; cnt signifies the centrosome. Bars: 4 μ m (a,b,e,f); 1 μ m (c); 100 nm (d,g).

7b/b',c/c') or multiply fragmented (Fig. 7d/d'). These data support the idea that the focal deformation of the prophase nuclear envelope is brought about by growing microtubules. Although fragmentation of the nuclear lamina can still occur in the absence of microtubules, the mode of nuclear envelope breakdown in nocodazole-treated cells differs significantly from the normal one and, apparently, involves different disassembly intermediates.

The physical continuity of the nuclear envelope is disrupted at late prophase/prometaphase

To assess whether the focal deformation of the nuclear envelope by growing microtubules results in mechanical disruption of the nuclear membrane, we examined Ishikawa cells that had been treated with low concentrations of the glucoside digitonin. This agent is known to permeabilize the cholesterol-rich plasma membrane, leaving the cholesterol-poor nuclear membrane intact (Griffiths, 1993). Under such circumstances, antigens located inside the nucleus (e.g. the lamins) are not accessible to exogenously added antibodies, unless the physical continuity of the nuclear envelope is disrupted. As seen in Fig. 8a/a', interphase cells and prophase cells up to the stage of the clepsydra did not have their lamina decorated by anti-lamin B antibodies. However, at late prophase/prometaphase, lamina fragments surrounding the chromosomes were clearly stained by anti-lamin B antibodies (Fig. 8b/b'). Consistent with the previous observations, at more advanced stages of mitosis lamin B was detected in the cytoplasm and at the center of the metaphase plate perpendicular to the spindle axis (Fig. 8c/c'). These data imply that the microtubule-induced deformation of the nuclear envelope is 'elastic' and does not result in the immediate piercing of the

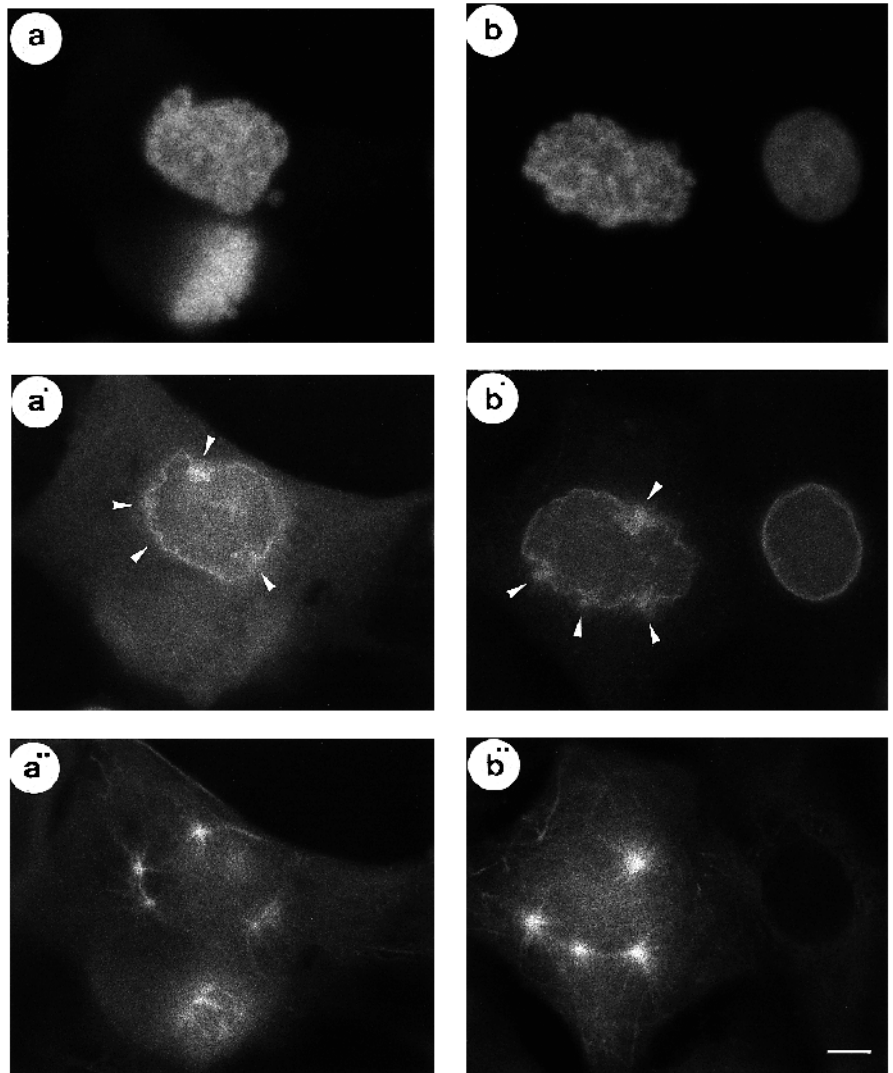


Fig. 5. Correlation of nuclear lamina indentations and microtubule asters. Ishikawa cultures were stained either with DAPI, anti-lamin A and anti-tubulin antibodies (a/a'/a''), or DAPI, anti-lamin B and anti-tubulin antibodies (b/b'/b''). The specimens were then surveyed to locate rare mitotic figures possessing more than the normal number of microtubule asters. Two such examples are shown here. Note the one-to-one correspondence between the microtubule asters and the focal indentations of the nuclear lamina (arrowheads). Bar, 3 μ m.

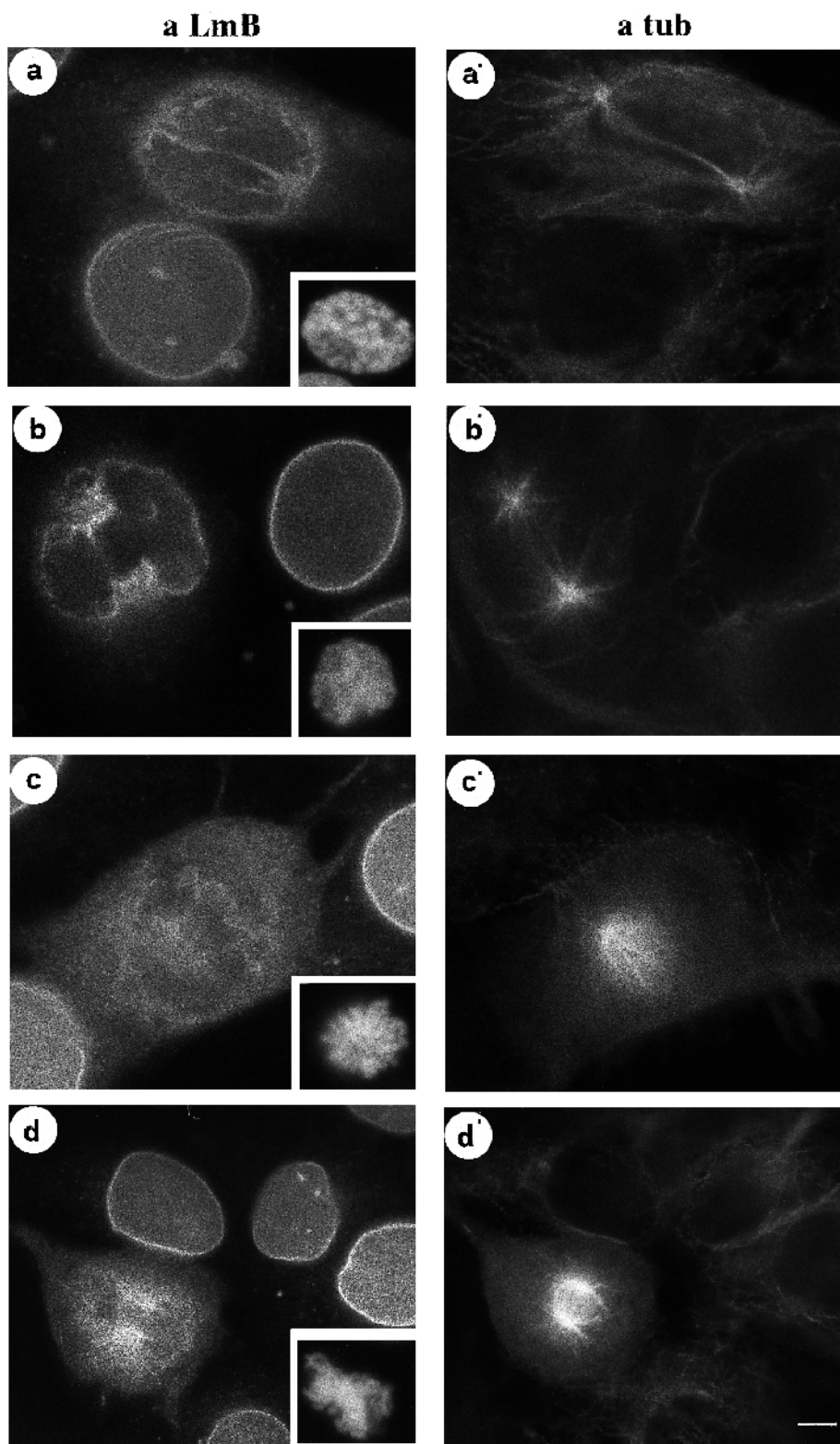


Fig. 6. Parallel monitoring of nuclear lamina disassembly and spindle assembly. Unsynchronized Ishikawa cultures were stained with anti-tubulin (a tub) and anti-lamin B antibodies (a LmB) and examined by conventional indirect immunofluorescence microscopy. The pairs a/a' and b/b' show successive stages of prophase; c/c' is a prometaphase and d/d' a metaphase cell. Bar, 3 μ m.

nuclear membrane. In other words, the disruption of the nuclear membranes seems to occur after A-type lamins have dissociated from the nuclear envelope and the remaining B-type lamina has partially fragmented.

The deduced sequence of events leading to nuclear envelope disassembly is shown schematically in Fig. 9.

DISCUSSION

Stepwise disassembly of the nuclear lamina

As noted in the Introduction, the mechanism of nuclear envelope disassembly during cell division remains elusive. Ultrastructural studies performed in the 1960s and 1970s have

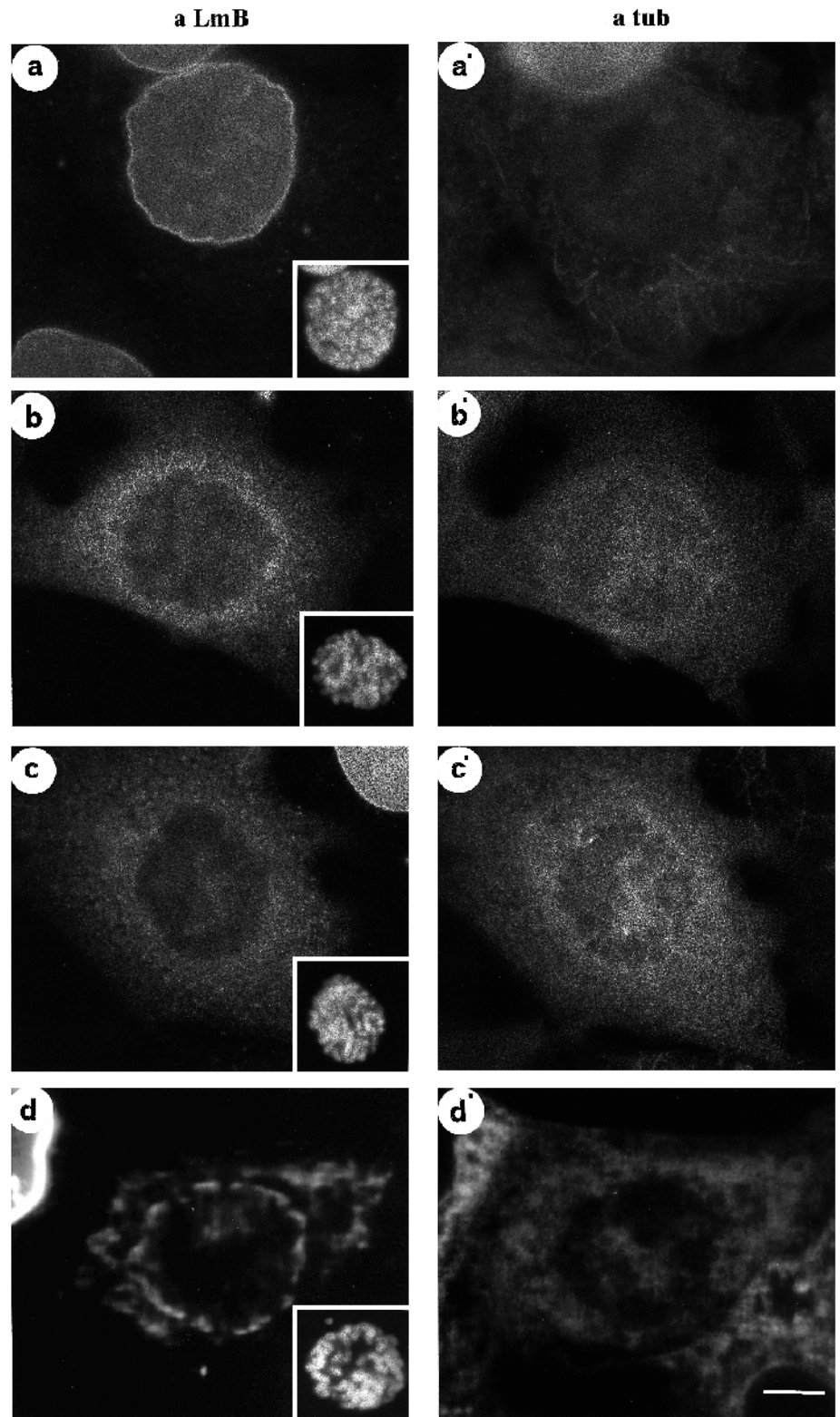


Fig. 7. Nuclear lamina disassembly in nocodazole treated cells. Unsynchronized Ishikawa cultures were exposed to nocodazole as specified in Materials and Methods. The cells were then stained with anti-tubulin (a tub) and anti-lamin B antibodies (a LmB) and visualized by conventional indirect immunofluorescence(a/a'-c/c') or confocal (d/d') microscopy. Notice that in the absence of microtubules the B-type lamina fragments differently from normal. Bar, 4 μ m.

revealed a series of orchestrated alterations occurring at the nuclear surface during the early phases of mitosis, before the complete breakdown of the nuclear envelope (Robbins and Gonatas, 1964; Murray et al., 1965; Bajer and Mole-Bajer, 1969; Ikeuchi et al., 1971; for a review see Hepler and Wolniak, 1984). However, these observations have been overshadowed

by subsequent work which suggested that nuclear envelope disassembly is a more or less 'cataclysmic' process, driven by mitotic hyperphosphorylation (reviewed by Nigg, 1992). The basis of this argument is the fact that phosphorylation by p34cdc2 results in lamin filament disassembly (Peter et al., 1991). Corroborating this point, sequence analysis of type A

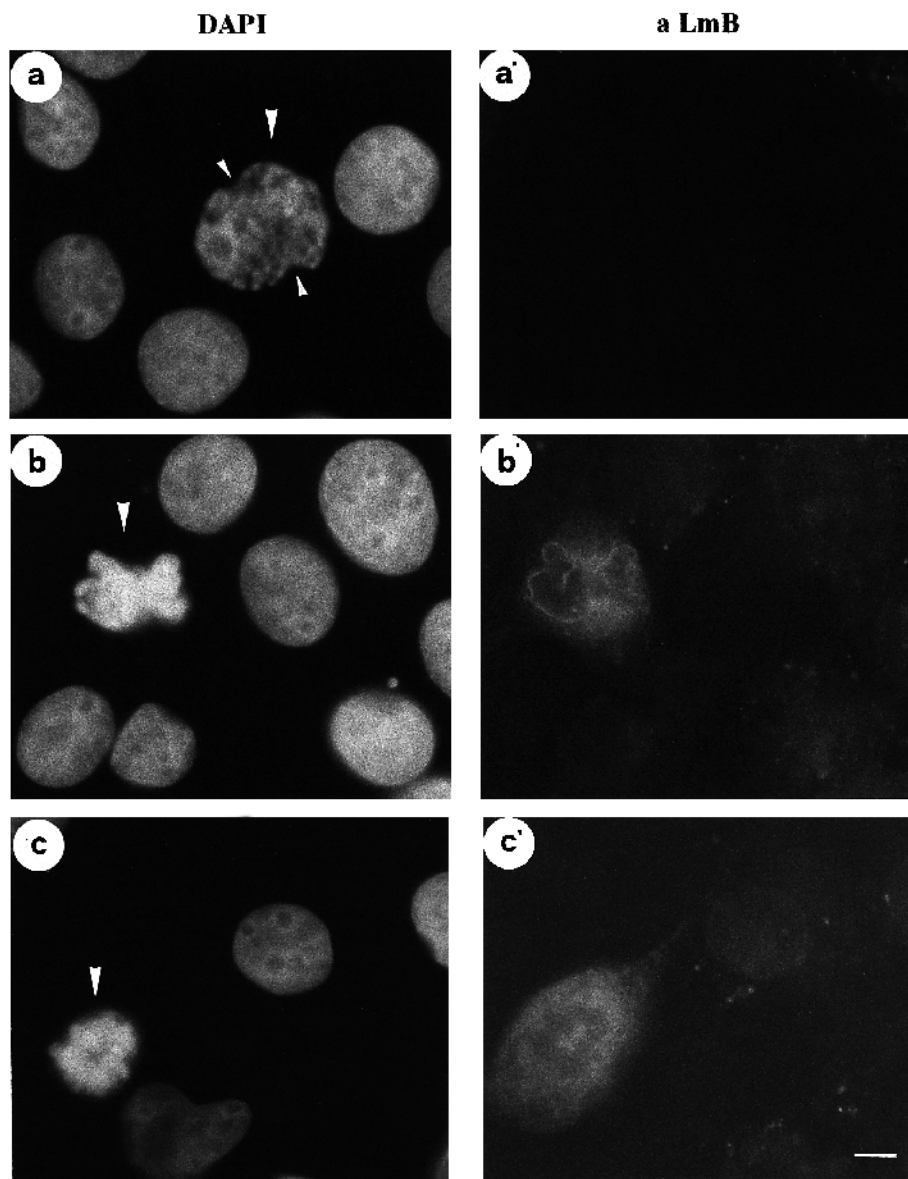


Fig. 8. Disruption of the nuclear envelope during late prophase/prometaphase. Unsynchronized Ishikawa cultures were permeabilized with digitonin, stained with DAPI and anti-lamin B antibodies (a LmB) and examined by conventional indirect immunofluorescence microscopy. (a/a') Mid-prophase; (b/b') late prophase/prometaphase; (c/c') metaphase. The lamina is not decorated until prometaphase, suggesting that the nuclear envelope is largely impermeable at the early stages of mitosis, despite the deformation (large arrowheads) of the nuclear lamina. Small arrowheads indicate mitotic cells present in each field. Bar, 3 μ m.

and type B lamins revealed the existence of consensus phosphorylation sites flanking the coiled-coil rod domain of these proteins, while analysis of mitotically disassembled lamins confirmed that they were indeed phosphorylated at such specific regions *in vivo* (Peter et al., 1990; Ward and Kirschner, 1990).

Although the significance of p34cdc2-mediated phosphorylation in the dissociation of interphase structures cannot be overemphasized, there is more to be said when one considers nuclear envelope disassembly in living cells. For instance, it is not clear *when* the lamin phosphorylation sites become accessible to p34cdc2, whether the corresponding sites of A- and B-type lamins are equally accessible at any given point during prophase/prometaphase and, in the final analysis, whether nuclear lamina disassembly is a catastrophic event (like microtubule shrinking) or a gradual, stepwise process.

The experiments reported here provide a first clue for a stepwise lamina disassembly. Although the disassembly schedules of A- and B-type lamins are temporally overlapping,

the former dissociate from the nuclear envelope earlier than the latter. Furthermore, careful inspection of early mitotic figures reveals that the disassembly patterns of the various lamin isotypes are distinct: the A-type lamin polymer depolymerizes fast during early/mid-prophase, yielding diffusible products; however, the B-type lamina is focally deformed and segmentally fragmented before its complete dismantling.

An important postulate of this work is that the deformation of B-type lamina does not involve blebbing and dissociation of the lamins from their inner nuclear membrane anchorage sites. Thus, irrespective of the weakening of lamin-lamin interactions, the nuclear envelope of early mitotic cells is still lined by a layer of B-type lamins, which might sustain the elasticity of the membrane and maintain the physical continuity of the nuclear envelope until prometaphase. Concerning the latter, it is noteworthy that, despite the deep indentations which develop at the surface of the nucleus in prophase, the nuclear envelope is not universally disrupted and does not succumb immediately to the pressure exerted by growing microtubules.

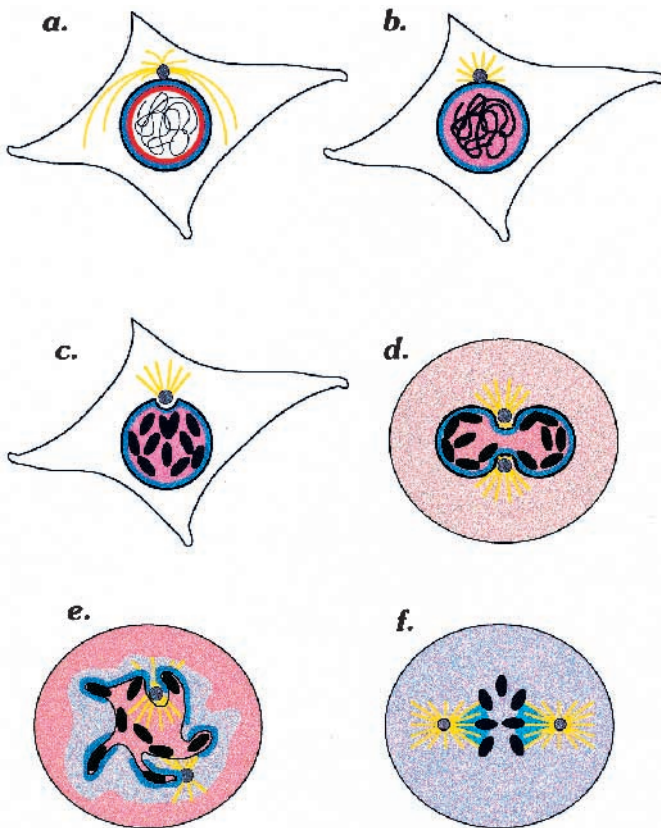


Fig. 9. A schematic representation of the stepwise disassembly of the nuclear envelope during mitosis. A-type lamins are indicated in red, B-type lamins in blue and microtubules in yellow. For details see Results.

Role of microtubules in nuclear envelope disassembly

We have shown that growing microtubules contribute to the orderly disassembly of the nuclear envelope. This contention is based on several lines of evidence. First, sister centrosomes and microtubule asters are accommodated exactly in the two nuclear indentations at early and mid-prophase. Second, microtubule growth towards the surface of the nucleus is closely correlated with the deepening of the nuclear indentations that culminates in characteristic clepsydra-like nuclei. Third, these focal deformations of the nuclear envelope and the nuclear lamina do not develop when the microtubules are disassembled by nocodazole.

Obviously, microtubule integrity is not necessary for nuclear lamina breakdown and membrane fragmentation, as cells incubated for a long period of time with nocodazole are finally arrested at prometaphase (Zieve et al., 1980) and end up with their lamina and nuclear envelopes disrupted (Burke and Gerace, 1986). However, we now understand that nuclear envelope breakdown in nocodazole-treated cells follows a disassembly stereotype that differs significantly from the normal one. Whether or not the sorting of nuclear membrane proteins and the fate of lamins are the same in unperturbed and nocodazole-treated cells remains to be determined.

Finally, it should be clarified that the focal indentations of

the prophase nucleus are distinct from other invaginations or channels that have been recently described in interphase cells (Fricker et al., 1997). In the former case, but not the latter, the deformations of the nuclear envelope develop at antidiapetric sites and are always associated with aster microtubules. Another difference between the two types of nuclear deformations is that the interphase invaginations transect the nucleus and are extremely narrow, whereas the indentations at prophase are originally wedge-shaped and only later develop into deep grooves. However, these two types of invagination do resemble each other in that both are lined by the nuclear lamina and the nuclear envelope membranes.

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