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The inner nuclear membrane protein LAP1 forms a native complex with B-type lamins and partitions with spindle-associated mitotic vesicles

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We have examined the *in situ* organization and nearest neighbours of the 'lamina-associated polypeptide-1' (LAP1), a type II membrane protein and a major constituent of the mammalian nuclear envelope. We show here that, during interphase, LAP1 forms multimeric assemblies which are suspended in the inner nuclear membrane and are specifically associated with B-type lamins. The LAP1–lamin B complex is distinct from analogous complexes formed by the 'lamina-associated polypeptide-2' (LAP2), another inner nuclear membrane protein, and includes a protein kinase. Upon nuclear envelope breakdown, LAP1 partitions with mitotic vesicles which carry nuclear lamin B. The LAP1 vesicles can be distinguished from fragments of the nuclear envelope containing LAP2 and exhibit a striking co-alignment with spindle microtubules. These observations suggest that the inner nuclear membrane comprises discrete territories which accommodate specific integral membrane proteins and are differentially disassembled during mitosis.

Keywords: integral membrane proteins/lamina-associated polypeptides/nuclear lamina

Introduction

The content of the cell nucleus is enclosed within a porous, membranous envelope. The nuclear envelope consists of three distinct membrane domains: the outer nuclear membrane, the pore membrane and the inner nuclear membrane. The outer membrane represents an extension of the rough endoplasmic reticulum (RER), while the inner nuclear membrane and the pore membrane possess unique characteristics (reviewed by Gerace and Burke, 1988; Georgatos, 1994; Goldberg and Allen, 1995; Wilson and Wiese, 1996).

Underlying the inner nuclear membrane is a meshwork of intermediate filaments, the nuclear lamina (Aebi *et al.*, 1986). This structure is a polymer of A- and B-type lamins and connects to the nuclear envelope via specific integral membrane proteins (for reviews, see Nigg, 1992; Georgatos *et al.*, 1994). The currently known lamin-binding proteins of the nuclear envelope include the lamin B receptor (LBR or p58; Worman *et al.*, 1988), the lamina-associated polypeptides (LAP1 and LAP2; Senior and Gerace, 1988; Foisner and Gerace, 1993) and a low molecular weight polypeptide termed p18 (Simos *et al.*, 1996).

LBR has been extensively characterized in mammalian and avian cells. It is a polytopic membrane protein possessing eight potential membrane-spanning regions and a long N-terminal segment exposed to the nucleoplasm (Worman *et al.*, 1990; Meier and Georgatos, 1994). This N-terminal part represents the 'business end' of the molecule and contains both lamin B (Ye and Worman, 1994) and chromatin (Pyrpasopoulou *et al.*, 1996; Ye and Worman, 1996) binding sites. LBR is modified post-translationally by a specific serine/arginine kinase, protein kinase A and p34/cdc2 (Appelbaum *et al.*, 1990; Courvalin *et al.*, 1992; Simos and Georgatos, 1992; Nikolakaki *et al.*, 1996, 1997). Interphase and mitotic phosphorylation modulate the interactions of LBR with its nearest neighbours and might provide a switch for its dynamic association with the lamina and the chromatin network (Appelbaum *et al.*, 1990; Nikolakaki *et al.*, 1996).

LAP2 is a type II integral membrane protein which belongs to the family of thymopoietins and is identical to thymopoietin β (Harris *et al.*, 1994; Furukawa *et al.*, 1995). Similar to LBR, this polypeptide is phosphorylated by interphase and mitotic kinases and exhibits lamin B and chromosome-binding properties *in vitro* (Foisner and Gerace, 1993).

Finally, the LAP1 includes three variants, LAP1A, 1B and 1C, which most probably represent differentially spliced products of the same gene (Senior and Gerace, 1988; Martin *et al.*, 1995). LAP1A and LAP1B have been reported to bind to lamin A/C and lamin B paracrystals, whereas LAP1C, the only LAP1 isoform whose amino acid sequence has been determined, does not have a lamin-binding activity *in vitro* (Foisner and Gerace, 1993). The properties of LAP1s, and especially the properties of LAP1C, are enigmatic. For instance, despite the lack of high affinity to the nuclear lamins under *in vitro* conditions, this protein constitutes the most abundant LAP1 isotype in a variety of cultured cells (Senior and Gerace, 1988; Martin *et al.*, 1995) and is likely to interact with components of the nuclear lamina. According to one report, LAP1C is capable of translocating from the inner nuclear membrane to the ER when the cells are in an undifferentiated state and A-type lamins are not expressed, but it becomes stably anchored at the nuclear envelope when

A-type lamins are incorporated into the lamina meshwork (Powell and Burke, 1990).

In higher eukaryotic cells, the nuclear envelope is disassembled completely during mitosis. This process involves lamina depolymerization and nuclear membrane breakdown (Suprynowicz and Gerace, 1986; Newport and Spann, 1987; Peter *et al.*, 1990). Recent studies suggest that LAP1s and LAP2 dissociate from the lamins at the onset of mitosis and reassemble around nascent nuclei before reformation of the nuclear lamina. However, in a somewhat paradoxical way, hyperphosphorylation of LAP2 abolishes binding to B-type lamins, whereas mitotically modified LAP1s are still capable of binding to A- and B-lamin paracrystals (Foisner and Gerace, 1993).

To resolve existing uncertainties, we have investigated systematically the spatial distribution and molecular interactions of the LAP1s during interphase and mitosis. We report here that under physiological conditions LAP1A/C are specifically bound to B-type lamins and a protein kinase. During mitosis, LAP1C and lamin B co-localize in a subpopulation of mitotic vesicles which associate transiently with the mitotic spindle and are sorted separately from mitotic vesicles containing LAP2. The implications of these findings in nuclear envelope organization and dynamics are discussed below.

Results

Production and characterization of anti-LAP1 antibodies

Initiating this study, we generated a series of monoclonal antibodies using as an antigen purified rat liver nuclear envelopes (for details, see Materials and methods). A subset of these antibodies reacted with the same doublet, i.e. two nuclear envelope proteins possessing molecular masses of 75 kDa and 55 kDa, respectively (Figure 1a and b; lanes NE). These polypeptides were not present in rat liver microsomes (Figure 1a and b; lanes ER), while the 55 kDa species was by far the most predominant in total homogenates of normal rat kidney (NRK) cells (Figure 1a and b; lanes NRK). Upon extraction of the nuclear envelopes with 8 M urea or 0.1 M NaOH, the antigens partitioned with the membrane-enriched, urea/alkali-insoluble fraction (Figure 1a and b; lanes urea and NaOH). Moreover, the 75 kDa and the 55 kDa protein could not be solubilized by 1% Triton X-100/20 mM Tris–HCl, but were removed from the nuclear envelopes by a combined 1% Triton X-100 and 150–500 mM NaCl treatment (Figure 1a and b; lanes TX and TX + salt).

The biochemical properties of the two antigens suggested an intimate association with the nuclear envelope and the nuclear lamina. To establish their identity, we immunoprecipitated material from salt and detergent extracts of rat liver nuclear envelopes and separated the two proteins by SDS–PAGE (Figure 1c). The 75 kDa and the 55 kDa bands were excised from preparative gels, digested with trypsin, and subjected to mass spectroscopy. As shown in Figure 1d, six unambiguous tryptic peptide sequences were obtained from the upper band and seven from the lower band. All peptides matched the reported amino acid sequence of a previously characterized protein, LAP1C, and were distributed along the entire length of this molecule (LAP1C residue numbers from the N- to the C-terminus: 8–22, 59–73, 127–148, 152–177, 241–259, 260–270, 373–393, 394–417 and 487–501; for sequence information see Martin *et al.*, 1995). Since three peptide sequences of the 75 kDa and the 55 kDa protein were identical, we deduced that the larger polypeptide corresponds to an isotype of LAP1C, LAP1A, while the smaller one corresponds to LAP1C itself. Although LAP1A/C and LAP1B (the third LAP1 isotype in mammalian cells) are supposedly similar in amino acid sequence and occur in comparable amounts in rat liver nuclear envelopes, the latter protein was not detected in immunoprecipitates of LAP1A/C (Figure 1c).

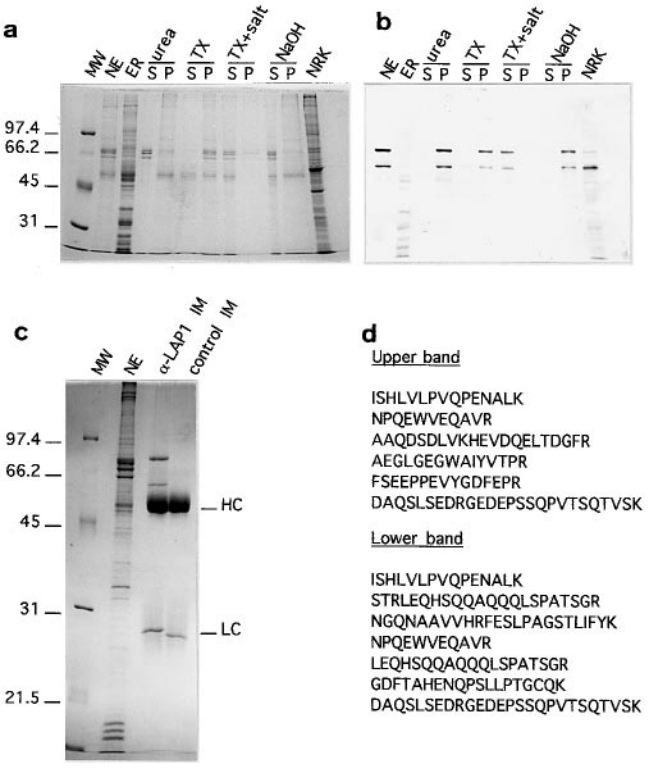

Fig. 1. Characterization of the anti-LAP1 antibodies. (**a**) Electrophoretic profiles of various subcellular fractions after SDS–PAGE and staining with Coomassie blue. MW, molecular weight markers with the indicated molecular masses in kDa; NE, rat liver nuclear envelopes; ER, rat liver microsomes; urea S/P, supernatant and pellet fraction after extraction of the nuclear envelopes with 8 M urea and 2 mM EDTA in 20 mM Tris–HCl, pH 7.4; TX S/P, supernatant and pellet fraction after extraction of the nuclear envelopes with 1% Triton X-100 in 20 mM Tris–HCl, pH 7.4; TX + salt S/P, supernatant and pellet fraction after extraction of the nuclear envelopes with 1% Triton X-100 and 0.5 M NaCl in 20 mM Tris–HCl, pH 7.4; NaOH S/P, supernatant and pellet fraction after extraction of the nuclear envelopes with 0.1 M NaOH; NRK, total homogenate of NRK cells. (**b**) Replica blot corresponding to the gel shown in (a). (**c**) SDS–PAGE profile of the material specifically precipitated by the mAbs (α-LAP1 IM) and a control mAb (control IM) from solubilized rat liver nuclear envelopes. MW are molecular weight markers with the indicated molecular masses in kDa; NE is a sample of salt-extracted rat liver nuclear envelopes; HC and LC denote the heavy and light chains of IgG, respectively. The gel has been stained with Coomassie blue. (**d**) Internal peptide sequences obtained from immunopurified 75 kDa and 55 kDa protein. For more details see Materials and methods.

Distribution of LAP1A/C in the interphase nucleus

When Triton X-100-permeabilized NRK cells were examined by indirect immunofluorescence, the anti-LAP1 antibodies decorated the surface of the nucleus in roughly

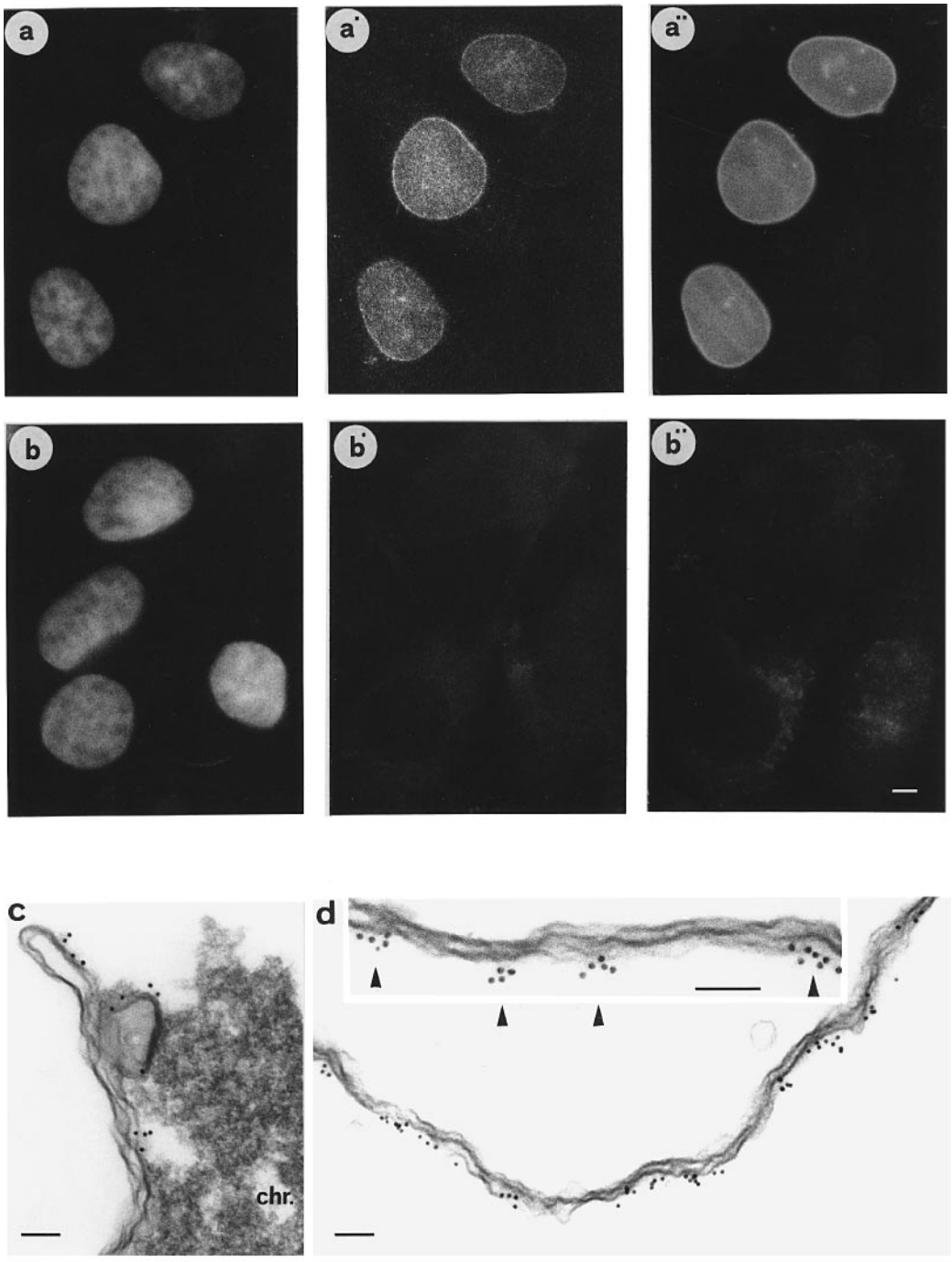

Fig. 2. Localization of LAP1A/C during interphase. (**a**, **a**′ and **a**″) Staining of Triton X-100-permeabilized NRK cells with DAPI, anti-LAP1 and anti-lamin B antibodies (in this order from left to right) as visualized by indirect immunofluorescence microscopy. (**b**, **b**′ and **b**″) Staining of digitonin-permeabilized NRK cells with DAPI, anti-LAP1 and anti-lamin B antibodies as visualized by indirect immunofluorescence microscopy. Bar = 2 μm. (**c** and **d**) Representative EM images of rat liver nuclear envelopes decorated by anti-LAP1 antibodies and 10 nm protein A–gold. Note the highly asymmetric distribution of LAP1A/C (arrowheads). 'chr' in (c) denotes a fragment of chromatin attached to the nucleoplasmic side of the nuclear envelope. Bars in (c), (d) and in inset to (d) correspond to 100 nm.

the same way as anti-lamin B antibodies (Figure 2a, a′ and a″). However, by comparison with the smooth rim fluorescence pattern of lamin B, the distribution of LAP1C (i.e. the predominant LAP1 variant in these cells; see Figure 1) was less uniform and notably punctate. To find out whether LAP1C was stably anchored at the inner nuclear membrane, or whether it partitioned between the inner and the outer nuclear membrane (for relevant data, see Powell and Burke, 1990; Simos *et al.*, 1996), we permeabilized NRK cells with the glucoside digitonin. Digitonin is known to permeabilize the cholesterol-rich plasma membrane, leaving intact the nuclear envelope. Thus, antigens exposed on the outer nuclear membrane and the ER are accessible to exogenously added antibodies, whereas antigens restricted to the inner nuclear membrane are not (Griffiths, 1993). When digitonin-treated cells were examined by double immunofluorescence microscopy, neither LAP1C nor lamin B were decorated (Figure 2b,

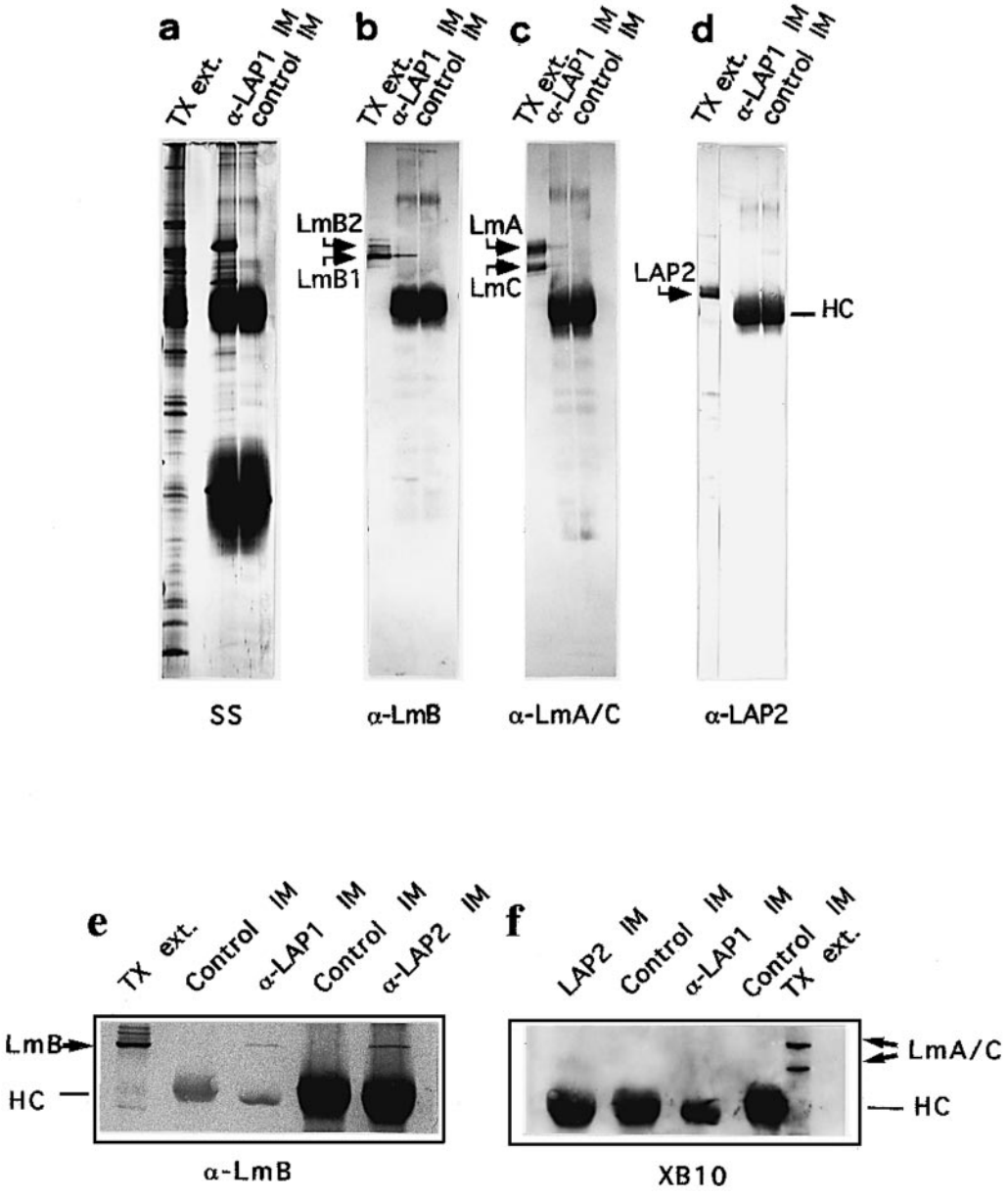

Fig. 3. Co-precipitation of B-type lamins with LAP1A/C. LAP1A/C and LAP2 were precipitated from solubilized rat liver nuclear envelopes by specific (α-LAP1 IM, α-LAP2 IM) or control (control IM) antibodies as specified in Materials and methods. TX ext. is a sample of the starting material used for immunoprecipitation. The samples were run in SDS–polyacrylamide gels and either stained with silver (**a**; SS), or probed with various antibodies, as indicated (**b**–**f**). α-LmB is an anti-peptide antibody developed against the tail domain of mouse lamin B1; α-LmA/C is an anti-peptide antibody raised against the head region of human lamins A/C; XB10 is a lamin A/C-specific mAb. The blot shown in (f) has been developed by ECL, while all other blots have been developed using conventional procedures. The positions of lamins B1, B2, A and C and the bands corresponding to LAP2 and the heavy chains of IgG (HC) are indicated.

b′ and b″). In contrast to this, similar treatment of NRK cells and staining with anti-tubulin or anti-vimentin antibodies revealed that cytoplasmic antigens were readily decorated (not shown). The conclusion that can be drawn from this experiment is that the entire LAP1 complement of NRK cells resides in the inner nuclear membrane.

Consistent with the indirect immunofluorescence data, analysis of rat liver nuclear envelope sections by immuno-electron microscopy showed that LAP1A/C were organized in clusters which were attached to the inner nuclear membrane (Figure 2c and d). The clustering of the antigens was not due to cross-linking induced by the bivalent antibodies, because the nuclear envelopes were fixed with formaldehyde before immunostaining. Moreover, this characteristic pattern of distribution has not been observed with other nuclear envelope proteins (e.g. p18) immunolocalized by the same method (Simos *et al.*, 1996).

Nearest neighbours of LAP1A/C

It has been reported previously that LAP1A and LAP1B (but not LAP1C) bind in a similar fashion to *in vitro* polymerized A- or B-type lamins, whereas LAP2 associates selectively with B-type lamins (Foisner and Gerace, 1993). To find out whether this reflects the *in vivo* situation, we precipitated LAP1A/C *en bloc* with associated proteins from freshly prepared 1% Triton X-100/150 mM NaCl extracts of rat liver nuclear envelopes. As depicted in Figure 3a–d, LAP1A/1C co-precipitated with B-type

lamins. However, only a negligible amount of lamins A/C and virtually none of the inner nuclear membrane protein LAP2 were detected in the precipitates. (In fact, the amount of lamins A/C seen in the blot of Figure 3c, lane α-LAP1 IM, represents the maximum quantity we have ever detected in such immunoprecipitation assays.) When LAP1C was precipitated from extracts of interphase NRK cells, lamin B could not be detected by regular immunoblotting. However, a small amount of lamin B specifically co-precipitating with LAP1C could be detected if a more sensitive detection system (ECL) was used (data not shown). From this we could tentatively conclude that both LAP1C and LAP1A possess a lamin B-binding activity.

In another series of experiments we employed, in addition to anti-LAP1 antibodies, an anti-peptide antibody against LAP2 (Pyrpasopoulou *et al.*, 1996). As could be expected, lamin B was readily co-precipitated with both LAP1A/C and LAP2 (Figure 3e). However, as shown in Figure 3f, the anti-LAP1 and anti-LAP2 antibody precipitates did not contain detectable amounts of A-type lamins, even when Western blotting was done using ECL. Furthermore, the same results were obtained when, instead of probing the blots with our own polyclonal antibodies, we utilized a monoclonal anti-lamin A/C antibody (XB10; kindly provided by Brian Burke). From the sum of these observations it can be safely concluded that both LAP1A/C and LAP2 interact specifically with B-type lamins. However, since neither LAP2, nor for that reason LAP1B, co-precipitate with LAP1A/C, it follows that the LAP1A/C–lamin B complex represents a distinct molecular ensemble separate from analogous complexes formed by LAP2 and other integral membrane proteins of the nuclear envelope.

Earlier studies have shown that another major protein of the inner nuclear membrane, LBR, forms an *in vivo* complex with the nuclear lamins and a serine/arginine kinase (Simos and Georgatos, 1992; Nikolakaki *et al.*, 1996). To find out whether the same paradigm holds for LAP1A/C, we immunoprecipitated the two proteins, incubated the precipitates with [γ-^{32}P]ATP, and analysed the products by SDS–PAGE and autoradiography. As shown in Figure 4a, several polypeptides, including a protein with the molecular mass of LAP1A and one with the molecular weight of LAP1C, were phosphorylated. Further experiments, presented in Figure 4b, indicated that the LAP1A/C-associated kinase activity did not dissociate from the complex upon washing with medium (150 mM) or elevated (500 mM) salt. In fact, under more stringent washing conditions the protein co-migrating with LAP1A was the only phosphorylated species detected by autoradiography (Figure 4b, lanes 5 and 6). The LAP1A/C-associated enzyme was sensitive to chelating agents and could be easily distinguished from casein kinase, protein kinase C and the LBR kinase, which is strongly inhibited by R1, a synthetic peptide representing a segment of the N-terminal region of LBR (Figure 4a; for pertinent information see Simos and Georgatos, 1992; Nikolakaki *et al.*, 1996).

Although the detailed characterization of the LAP1A/C-associated kinase was outside the scope of this study, we found it important to clarify whether it phosphorylated serine/arginine motifs, similarly to the LBR kinase. To approach this question, we repeated the *in vitro* phosphorylation experiments in the presence and absence of the synthetic peptide R0 which contains multiple serine/arginine repeats and inhibits specifically the serine/arginine kinases (Nikolakaki *et al.*, 1996). As depicted in Figure 4b (compare lanes 1 and 2 with lanes 5 and 6), the LAP1A/C-associated kinase was not affected by R0. From these data it should be concluded that the enzyme does not belong to the family of the serine/arginine kinases.

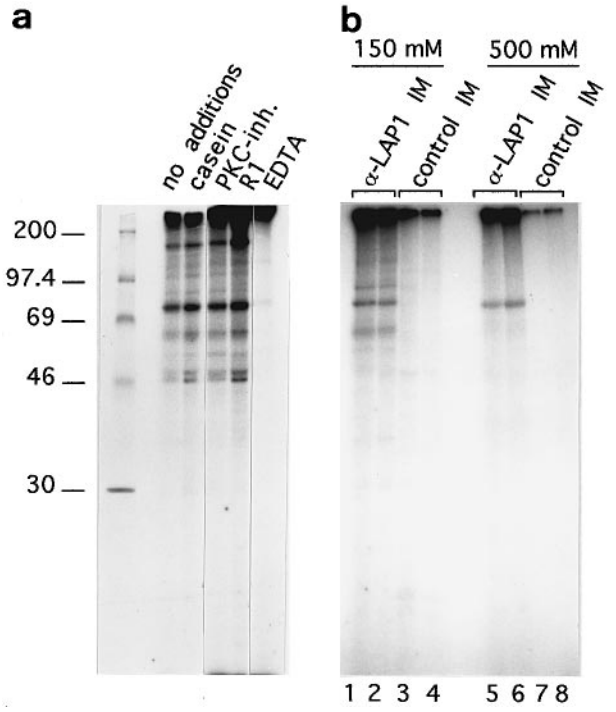

Fig. 4. LAP1A/C are associated with a protein kinase. (**a**) Material precipitated by anti-LAP1 antibodies was resuspended in 0.1% Triton buffer containing 50 μM [γ-^{32}P]ATP (2000 Ci/mmol), incubated for 60 min at 30°C and processed for SDS–PAGE and autoradiography. *In vitro* phosphorylation reactions were done in plain buffer (no additions), or in the presence of 400 μg/ml casein, 10 μM of protein kinase C inhibitor (PKC-inh.), 0.5 mM of peptide R1, or 5 mM EDTA. ^{14}C-labelled molecular weight makers are shown on the left of the autoradiogram. (**b**) A similar *in vitro* phosphorylation experiment in which the immunoprecipitates were washed with 150 mM (lanes 1–4), or 500 mM NaCl (lanes 5–8). In samples 2, 4, 6 and 8 the assay mixture contained 4 mM of peptide R0 (SSPSRRSRSRSRS-RSPGRPAKG; single-letter amino acid code), whereas in samples 1, 3, 5 and 7 there were no additions. Antibody designations are as in Figure 3. For further details see Materials and methods.

Partitioning of LAP1C in naturally occurring mitotic cells

To monitor the distribution of LAP1C during mitosis, we stained cultures of NRK cells with the corresponding antibodies and surveyed the specimens with confocal microscopy. Although a fraction of LAP1C-containing particles (presumably mitotic vesicles) were found to be scattered in the mitotic cytoplasm, there was an obvious accumulation of the antigen at specific subcellular locales. This was particularly evident in optical sections normal to the metaphase plate, which indicated that LAP1C was highly concentrated in the area of the (presumed) mitotic spindle (Figure 5a). During anaphase, LAP1C was more concentrated on the surfaces of chromosomes, especially on the sides facing the spindle poles (Figure 5b). Finally,

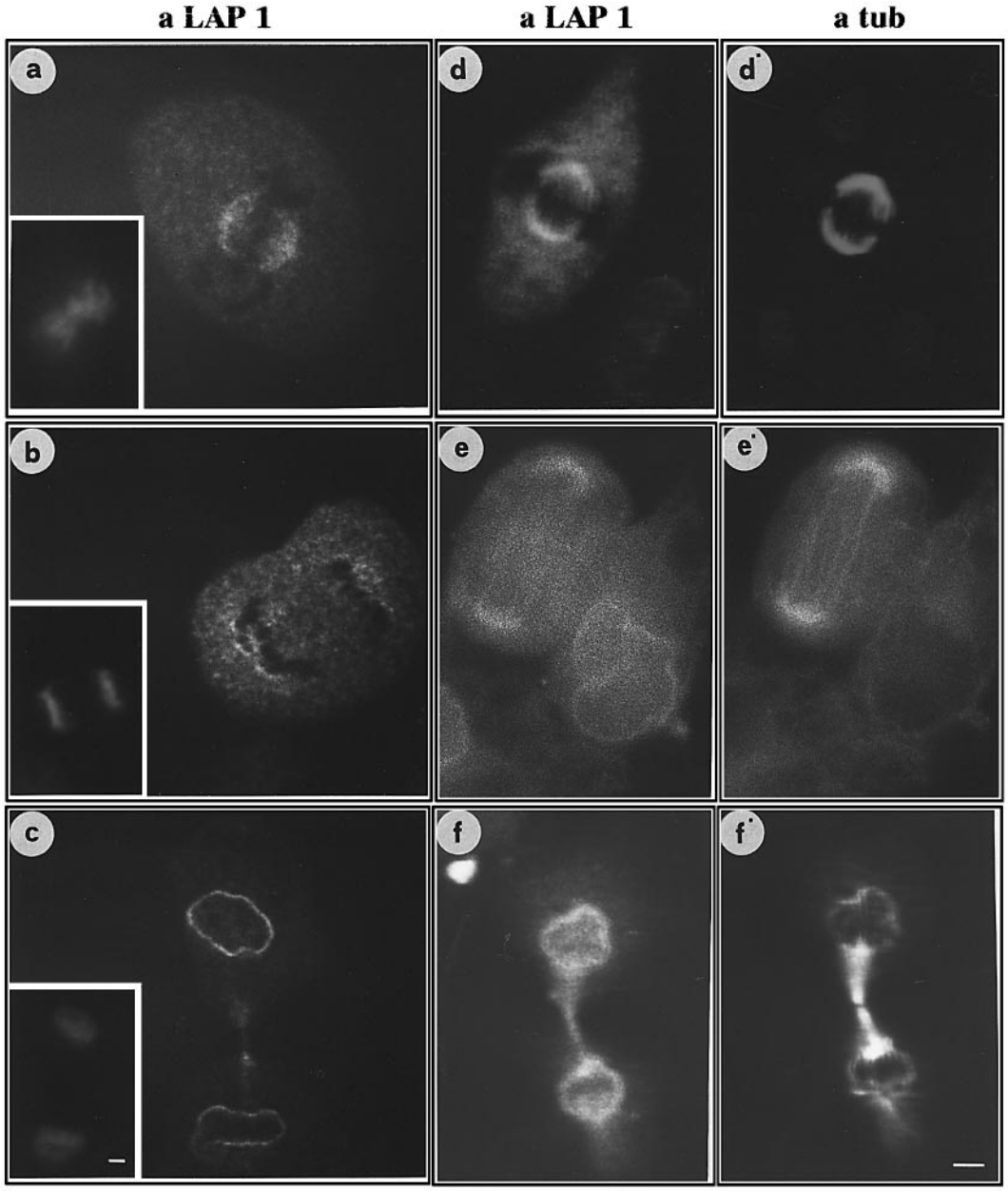

Fig. 5. LAP1C co-localizes with the mitotic spindle. (**a**–**c**) Naturally occurring mitotic cells from an NRK culture were fixed with formaldehyde, permeabilized with Triton X-100 and stained with anti-LAP1 antibodies and DAPI (insets). (**d** and **d′**, **e** and **e′** and **f** and **f′**) Mitotic NRK cells doubly labelled with the mAbs to LAP1 (a LAP1) and polyclonal antibodies to α-tubulin (a tub). All images, except (e and e′), have been recorded at the confocal microscope. (a, d, and d′), metaphase; (e and e′), late anaphase; (f and f′), late telophase/G_1. Notice the highly asymmetric distribution of LAP1C and the close association with the mitotic spindle. Bars = 2 μm.

in late telophase/cytokinesis, although the bulk of the antigen had reassembled around the two daughter nuclei, a clearly discernible signal could be seen in the region of the mid-body (Figure 5c). As the anti-LAP1 antibodies did not decorate the microtubules or other cytoplasmic structures and reacted exclusively with LAP1 proteins in both interphase and mitotic cells, we inferred that a fraction of LAP1C-containing vesicles should be closely associated with the mitotic spindle.

Pursuing this idea, we performed double-labelling experiments using anti-LAP1 and anti-tubulin antibodies. As shown in Figure 5d, d′, e and e′, the LAP1C fluorescence pattern was strikingly similar to the microtubule pattern during metaphase and anaphase. From that stage on, the two patterns became distinct, as LAP1C reassembled around chromosomes, while the microtubules concentrated at the mid-body region. Nevertheless, at all specimens examined there was discernible LAP1C staining in the area accommodating the mid-body (Figure 5f and f′). Confirming a specific interaction between LAP1C-containing vesicles and the mitotic apparatus, examination of nocodazole-treated NRK cells revealed that LAP1C is no longer 'focused' at a specific region when the microtubules are depolymerized (data not shown).

To find out whether the association of LAP1C vesicles with the mitotic spindle was unique, or whether other

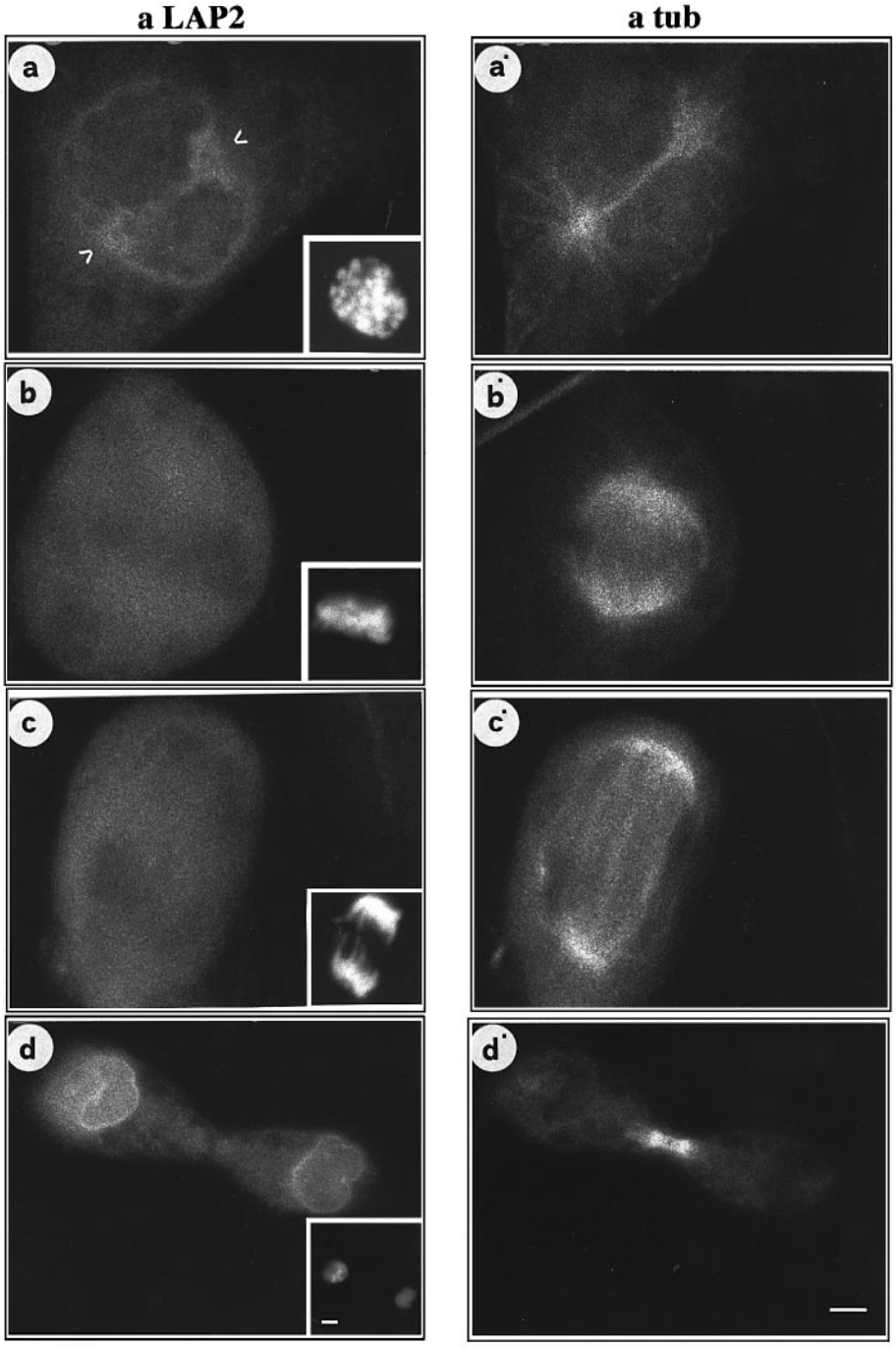

Fig. 6. Distribution of LAP2 in mitotic cells. Naturally occurring mitotic cells were processed as in Figure 5 and stained with polyclonal antibodies to LAP2 (a LAP2) and a monoclonal antibody to α-tubulin (a tub). DAPI staining is shown in insets. All images have been recorded in a conventional fluorescence microscope. (**a** and **a′**), prophase; (**b** and **b′**), metaphase; (**c** and **c′**), anaphase; (**d** and **d′**), late telophase/G_1. Arrowheads in (a) indicate the two symmetrical indentations which develop opposite to microtubule asters in all prophase cells. Bars, 2 μm.

inner nuclear membrane proteins were sorted in the same way during mitosis, we double-stained NRK cells with anti-LAP2 and anti-tubulin antibodies. As shown in Figure 6, the distribution of LAP2 did not match the microtubule pattern and differed markedly from that of LAP1C. In particular, the dissociation of LAP2 from the nuclear envelope appeared to start at mid-prophase (Figure 6a and a′), when the nuclear envelope develops two antidiametrical invaginations and LAP1C–lamin B are still in an assembled state (see below). At metaphase, the bulk of LAP2 was scattered throughout the cytoplasm (Figure 6b and b′) and remained in this diffuse state for the most part of anaphase (Figure 6c and c′). However, LAP2 reassembled fully around the two daughter nuclei by late anaphase–telophase (Figure 6d and d′). The same results were obtained when we studied the mitotic distribution of LAP2 in Ishikawa cells, a human endometrial carcinoma cell line (data not shown).

To establish that LAP1 and LAP2 were sorted differently during mitosis, we double-labelled NRK cells with the

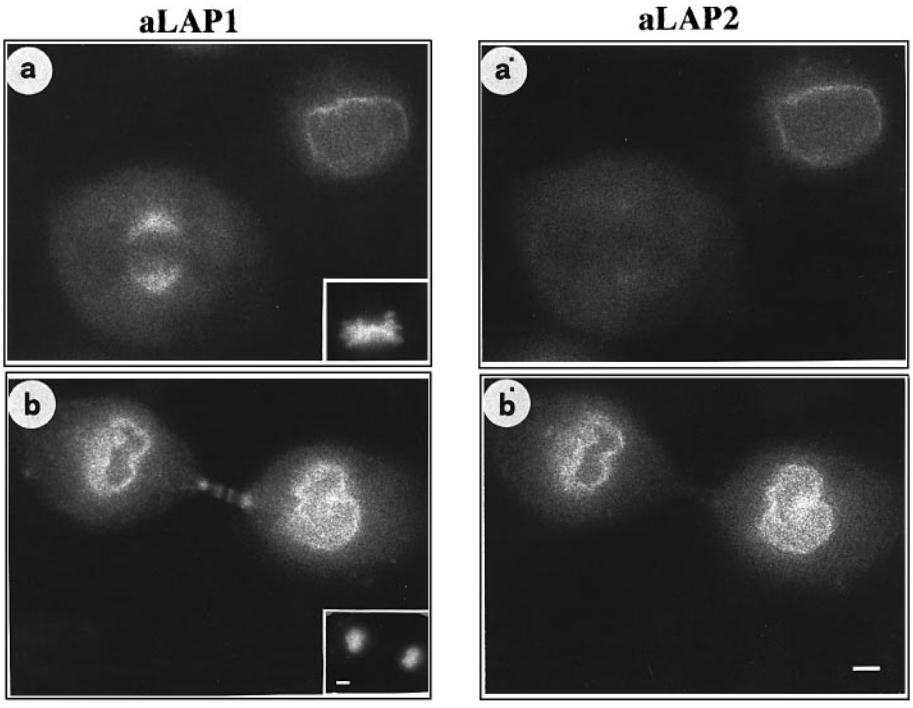

Fig. 7. LAP1C and LAP2 partition differently during mitosis. Naturally occurring mitotic cells were processed as previously and stained with mAbs to LAP1 (aLAP1) and polyclonal antibodies to LAP2 (aLAP2). DAPI profiles are shown in insets. All images have been recorded in a conventional fluorescence microscope. (**a** and **a**′), metaphase; (**b** and **b**′), late telophase/G_1. Bars = 2 μm.

corresponding antibodies. As shown in Figure 7, the two proteins exhibited distinct distribution patterns during metaphase and late telophase/early G_1, i.e. at the two stages where the co-localization of LAP1C with the mitotic spindle and the mid-body microtubules is conspicuous (see Figure 5).

To compare the distribution of LAP1C with that of lamin B, we double-labelled NRK cells with the corresponding antibodies. At early prophase (Figure 8a and a′), the LAP1C and lamin B were co-aligned along the characteristic invaginations which develop opposite to the microtubule asters and herald the onset of nuclear envelope disassembly (S.D.Georgatos *et al.*, 1997). At metaphase (Figure 8b and b′), both proteins concentrated in the spindle region and overlapped extensively. At anaphase (Figure 8c and c′) LAP1C and B-type lamins were localized on the surfaces of the chromatin packages facing the spindle poles. Finally, as anticipated, by telophase (Figure 8d and d′) LAP1C and lamin B had reassembled around the nascent nuclear envelopes. These experiments support the idea that the bulk of LAP1C and lamin B reside in the same mitotic vesicles and follow the same fate during cell division. However, since we have never observed lamin B staining corresponding to the mid-body, as with LAP1C, it is obvious that a fraction of LAP1C vesicles do not contain lamins.

To ascertain that LAP1C and B-type lamins resided in the same mitotic vesicles, we performed biochemical experiments using synchronized, prometaphase cells. LAP1C-containing vesicles were immunoisolated by incubating homogenates of nocodazole-arrested cells with magnetic beads that carried anti-LAP1A/C antibodies (for technical details see Maison *et al.*, 1993, 1995). The material retained by the immunomatrices was then analysed by Western blotting using a set of specific antibodies. Figure 9a shows that the immunoisolation of mitotic vesicles was specific because the LAP1C protein was captured only by the anti-LAP1 immunobeads and not by the beads carrying control antibodies. LAP1C vesicles isolated from nocodazole-arrested cells also contained lamin B (Figure 9c) and were associated with the intermediate filament protein vimentin (Figure 9b). The physical interaction of vimentin filaments with vesicles containing lamin B is in line with previously reported observations on nocodazole-arrested Chinese hamster ovary cells (Maison *et al.*, 1993, 1995).

Discussion

Integral proteins of the inner nuclear membrane organize as distinct junctional complexes

The observations reported here provide a new example of a multimeric complex which includes integral membrane proteins of the nuclear envelope, the nuclear lamins and a protein kinase. A precedent for such a transmembrane assembly has been the LBR complex, a large macromolecular formation which comprises LBR, the nuclear lamins, a serine/arginine kinase and several other components (Simos and Georgatos, 1992, 1994; Nikolakaki *et al.*, 1996).

The existence of a LAP1A/C complex at the inner nuclear membrane is consistent with the clustered distribution of these proteins *in situ*, the tight binding of LAP1A/C to B-type lamins, and the co-distribution of LAP1C and lamin B during mitosis. Although LAP1A binds both type A- and B-type lamins *in vitro* (Foisner

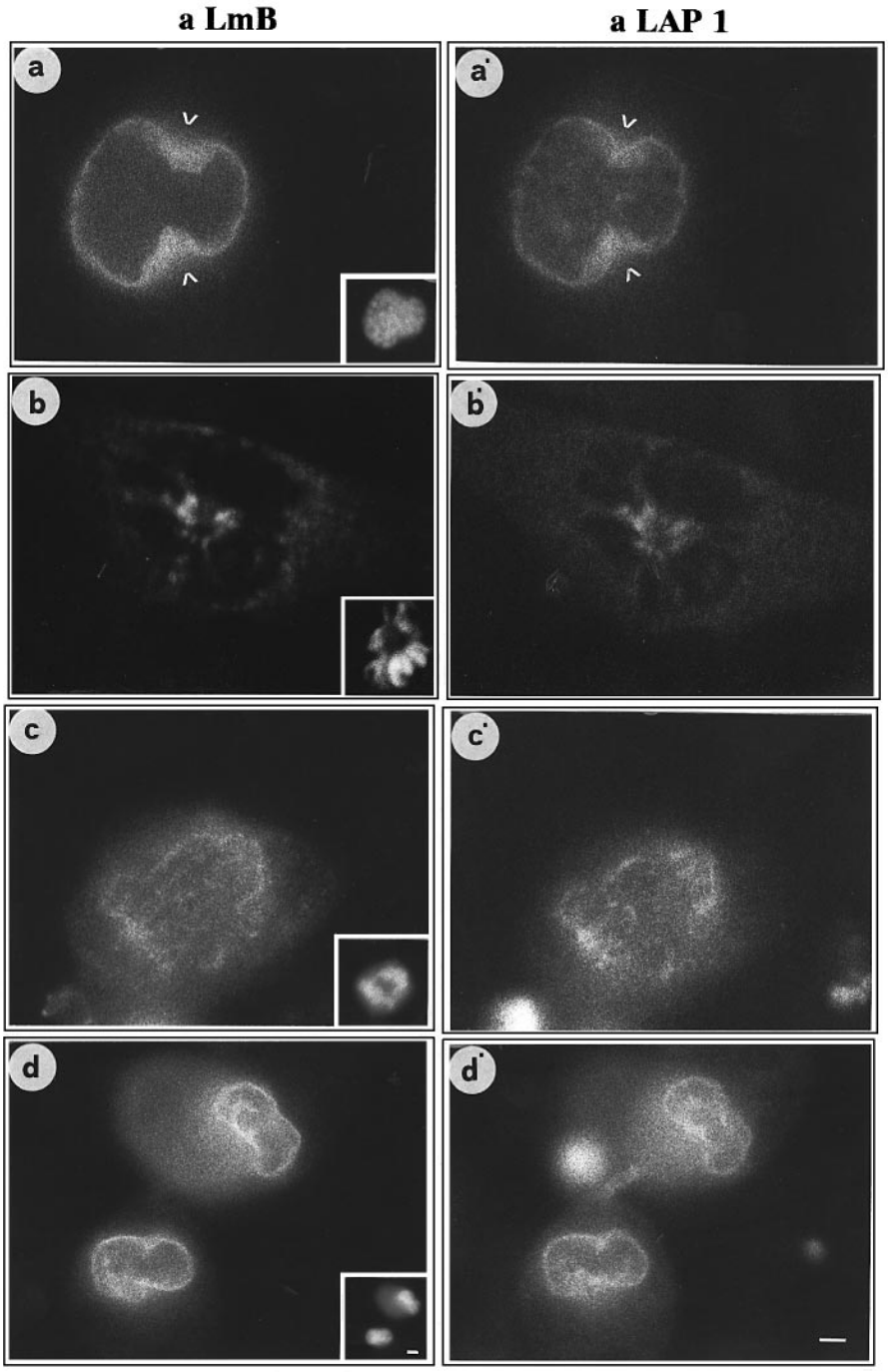

Fig. 8. LAP1C co-segregates and co-assembles with B-type lamins during cell division. Naturally occurring mitotic cells doubly labelled with polyclonal antibodies to lamin B (aLmB) and mAbs to LAP1 (a LAP1). Insets show DAPI staining. All pictures, except (b and b′), have been taken in a conventional fluorescence microscope. (b and b′) has been recorded in a confocal microscope. (**a** and **a**′), prophase; (**b** and **b**′), metaphase (viewing direction parallel to the spindle axis, perpendicular to the metaphase plate); (**c** and **c**′), anaphase; (**d** and **d**′), late telophase. Note the similarity in the distribution patterns at all phases of mitosis. Arrowheads in (a) and (a′) indicate the two symmetrical indentations on the surface of the prophase nucleus. LAP1 and lamin B remain well localized during prophase. Bars = 2 μm.

and Gerace, 1993), LAP1A/C extracted under native conditions from rat liver nuclear envelopes do not co-precipitate with A-type lamins. There can be two interpretations for this: (i) either A-type lamins dissociate from LAP1A/C during membrane solubilization; or (ii) these polypeptides are not physically interacting *in situ*. In either case, it would appear that A-type lamins are not an integral part of the 'core' LAP1A/C assembly.

Since the molecular architecture and the exact stoichiometry of the LAP1A/C–lamin B complex is not yet known, it is not clear whether the nucleoplasmic domains of several LAP1A/C molecules are interconnected by B-type

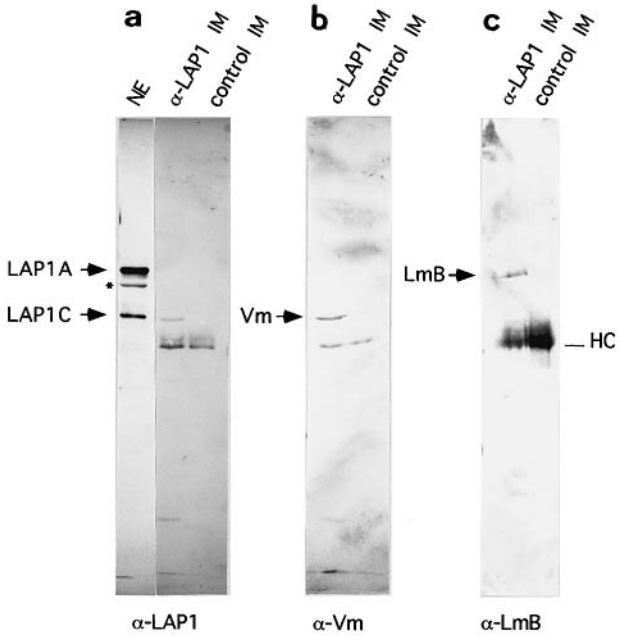

Fig. 9. Co-isolation of LAP1C, B-type lamins and vimentin from mitotic homogenates of nocodazole-arrested cells. NRK cultures were synchronized with 50 ng/ml nocodazole and loosely attached prometaphase cells were recovered by a shake-off. After mechanical lysis, the resulting homogenates were incubated with magnetic beads carrying either anti-LAP1, or control antibodies. Following washings with assay buffer, the elements bound to the beads were resolved by SDS–PAGE and analysed by Western blotting (for technical details see Materials and methods and Maison *et al*., 1993; 1995). (**a**) Material immunoabsorbed by anti-LAP1 (α-LAP1 IM) and control (control IM) antibodies, together with a sample of salt-washed rat liver nuclear envelopes (NE), probed with anti-LAP1 antibodies. Asterisk marks a degradation product of LAP1A. (**b** and **c**) Similar blots stained with anti-vimentin and anti-lamin B antibodies, respectively. The positions of LAP1A/1C, vimentin (Vm), lamin B (LmB) and the heavy chains of IgG (HC) are indicated.

lamin oligomers or whether an oligomeric 'core' unit is organized by lateral binding of adjacent transmembrane regions of LAP1A/C.

Sorting of LAP1 and LAP2 during mitosis

The existence of organized complexes at the inner nuclear membrane implies that the major lamin-binding proteins of the nuclear envelope (e.g. LAP1s, LAP2, LBR) do not intermingle and may separate from each other upon mitotic disassembly of the nuclear envelope. In line with this idea, LAP1A/C do not co-precipitate with LAP2 or LAP1B, although all of these proteins are associated with B-type lamins. In addition, LAP1C and LAP2 are sorted differently during mitosis.

The packaging of various nuclear envelope proteins into different mitotic vesicles does not necessarily require 'specificity' and might be the direct consequence of their oligomeric organization and/or clustered distribution. Assuming that nuclear envelope vesiculation occurs in a stochastic fashion (Warren, 1993), it is apparent that vesicles containing small pieces of the inner nuclear membrane would be automatically enriched in whatever component happened to be most abundant in the territory from which they originated. If this area happened to contain a LAP1A/C cluster, a particular vesicle would contain a high proportion of LAP1A/C and a low percentage of other integral membrane proteins. With the same logic, a vesicle originating from an adjacent region would be depleted in LAP1A/C, because the distribution of the clusters is discontinuous, and enriched in other membrane proteins. The differential sorting of inner nuclear membrane proteins during mitosis might explain why various lamin B isotypes are distributed in different subpopulations of mitotic vesicles as has been reported to be the case in the *Xenopus* oocyte (Lourim and Krohne, 1994; Lourim *et al.*, 1996). Moreover, the idea that clustered nuclear envelope proteins are segregated away from one another during mitosis fits the fact that integral membrane proteins of the nuclear pore complex (e.g. gp210) disassemble and reassemble independently of inner nuclear membrane proteins during cell division (Chaudhary and Courvalin, 1993).

In a previously published study (Foisner and Gerace, 1993) it has been reported that the distribution of LAP1/LAP2 in mitotic NRK cells differs from that of B-type lamins, the latter being in a diffuse state from metaphase to telophase (Foisner and Gerace, 1993). We could not confirm this using our methods in any of the cell lines we have examined (NRK, CHO, DU249 cells). However, we feel that the seemingly diffuse distribution of nuclear lamin B might be related to the fixation protocol employed. We have noticed that acetone/methanol-fixed mitotic cells, or even aldehyde-fixed specimens that have been left for days at 4°C, are sometimes stained weakly or indistinctly with anti-lamin B antibodies (for a relevant comment, see Meier and Georgatos, 1994). Therefore, it is likely that mitotically modified lamins are partially solubilized by these solvents instead of being properly fixed.

Dynamic interactions between nuclear envelope fragments and elements of the cytoskeleton

Using Chinese hamster ovary (CHO) cells synchronized at prometaphase with microtubule-depolymerizing agents, we have found previously that vesicles carrying B-type lamins are tightly connected to vimentin intermediate filaments (Maison *et al.*, 1993, 1995). The same seems to hold for mitotic NRK cells, since LAP1C, B-type lamins and vimentin are co-immunoisolated from total homogenates of nocodazole-arrested cells (this report). However, in naturally occurring mitotic cells, which possess an intact microtubule system, the bulk of lamin B–LAP1C vesicles are clearly associated with the mitotic spindle. The interpretation we offer to explain these findings is that lamin B-carrying fragments of the nuclear envelope probably bind to one or more microtubule and intermediate filament-associated proteins (MAPs/IFAPs). An illustrative example of such a protein is plectin (Wiche *et al.*, 1991). This polypeptide is known to bind nuclear lamin B (Foisner *et al*., 1991) and has been recently found to cross-link vimentin filaments with other cytoskeletal elements, including the microtubules (Svitkina *et al*., 1996). It could be that plectin or a related protein are massively released from the microtubules upon nocodazole treatment, switching entirely to intermediate filaments and creating an array of new binding sites for mitotic vesicles on the surfaces of these elements.

Irrespective of these interpretations, the important point emerging from current and previous studies is that the cytoskeleton plays an active role in membrane partitioning

during cell division. This idea is in excellent agreement with previous ultrastructural studies. For instance, earlier work has revealed intimate connections between aster microtubules and the nuclear envelope during prophase and prometaphase (Bajer and Mole-Bajer, 1969; for relevant data see also Murray *et al.*, 1965), while membranous structures and chains of vesicles radiating along microtubule asters have been seen in living cells and in isolated mitotic apparatus (reviewed in Hepler and Wolniak, 1984). With regard to that, a particularly interesting new finding is that a fraction of LAP1C is still attached to mid-body microtubules during late telophase/early G_1.

Materials and methods

Cell culture and synchrony

NRK cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. Cells were synchronized at prometaphase with 50 ng/ml nocodazole for 20–24 h. To disrupt the microtubule system in unsynchronized cultures, the cells were chilled on ice for 30 min and then incubated in the presence of 33 μM nocodazole for 30 min at 37°C.

Antibodies

The anti-LAP1 monoclonal antibodies C10, C7, B11 and E3 were produced and isolated according to standard procedures. All antibodies recognized LAP1A and LAP1C and were used as a mixture for probing blots or staining cells in culture. The rabbit anti-LAP2 antibody (Pyrpasopoulou *et al.*, 1996) was raised against the peptide QALTREST-RGSRRTPRRRVEK which corresponds to a stretch of the N-terminal domain of this protein (for sequence information, see Furukawa *et al.*, 1995). The anti-lamin peptide antibodies No.16 (lamin B-specific), and aLI (anti-lamin A/B/C-specific at low dilutions and anti-lamin A/C-specific at high dilutions) have been described in previous studies (Simos and Georgatos, 1992; Maison *et al.*, 1993, 1995; Meier and Georgatos, 1994; Pyrpasopoulou *et al.*, 1996). The monoclonal antibody XB10 which recognizes specifically lamins A/C was a generous gift of Brian Burke. A monoclonal and a polyclonal antibody developed against the erythrocyte-specific nuclear envelope protein p18 (Simos *et al.*, 1996) were used as controls throughout this study. Monoclonal antibodies against α- and β-tubulin were purchased from Sigma and Amersham. A polyclonal antibody against α-tubulin was obtained from E.Karsenti (EMBL, Heidelberg, Germany). The monoclonal anti-vimentin antibody 7A3 (Papamarcaki *et al.*, 1991) and the rabbit anti-peptide antibody aV2 (Maison *et al.*, 1993) were used as needed in various control experiments.

Biochemical and immunochemical procedures

Isolation of rat liver nuclear envelopes, fractionation of mitotic cells, *in vitro* phosphorylation assays, immunoblotting and peptide sequencing were performed as described previously (Simos *et al.*, 1992, 1996; Maison *et al.*, 1993; Nikolakaki *et al.*, 1996; Pyrpasopoulou *et al.*, 1996). For immunoprecipitation experiments, we used two different protocols. According to the first protocol, rat liver nuclear envelopes or NRK cells were extracted with buffer S1 (1% Triton X-100, 20 mM Tris–HCl, pH 7.2, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM PMSF) for 30 min on ice. The clarified supernatant (12 000 *g*, 20 min, 4°C) was incubated with monoclonal antibodies and protein A–Sepharose preincubated with rabbit anti-mouse IgG for a total of 3 h at 4°C. The immune complexes were recovered by centrifugation (12 000 *g*, 2 min, 4°C) washed three times with regular buffer S1 and once with buffer S1:0.1% Triton X-100, and solubilized in hot sample buffer. Alternatively, rat liver nuclear envelopes were extracted with buffer S2 (2% Triton X-100, 40 mM Tris–HCl, pH 7.5, 300 mM NaCl, 5 mM DTT, 10% sucrose, 1 mM EGTA, 1 mM PMSF and 2 μg/ml leupeptin, apronitin, antipapain) for 10 min on ice. The extract was clarified by centrifugation (12 000 *g*, 15 min at 4°C), diluted 1:1 with 10% sucrose in double-distilled water and dialysed for 2–4 h at 4°C against buffer S1 to remove DTT. After another centrifugation step (12 000 *g*, 30 min at 4°C), the supernatant was incubated for 1 h with monoclonal antibodies and protein G–Sepharose or rabbit antibodies and protein A–Sepharose on ice. The immune complexes were recovered by centrifugation, washed five times with buffer S1 and once with PBS and solubilized in hot sample buffer. Both protocols yielded the same results; however, extraction of membrane proteins was more efficient when buffer S2 was used. *In vitro* phosphorylation assays were done in a buffer containing 0.1% Triton X-100, 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$ and 1 mM DTT supplemented with 50 μM [γ-^{32}P]ATP.

Fluorescence and electron microscopy

Conventional and confocal fluorescence microscopy and electron microscopy were done as described previously (Maison *et al.*, 1993, 1995; Meier and Georgatos, 1994). The samples were fixed with 4% formaldehyde in PBS (10 min, room temperature). Under standard conditions, fixed cells were permeabilized with 0.5% Triton X-100 in PBS and non-specific antibody binding sites were blocked by incubation with 1% fish skin gelatin which was present in all media except the fixative. When needed, cells were permeabilized with 0.002% digitonin in PBS for 2–5 min at room temperature before fixation. The efficiency and specificity of digitonin permeabilization were regularly checked by double staining with anti-vimentin/anti-tubulin (cytoplasmic markers) and anti-lamin B (nucleoplasmic marker) antibodies.

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