Detergent-salt resistance of LAP2 α in interphase nuclei and phosphorylation-dependent association with chromosomes early in nuclear assembly implies functions in nuclear structure dynamics

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Lamina-associated polypeptide (LAP) 2 of the inner nuclear membrane (now LAP2 β) and LAP2 α are related proteins produced by alternative splicing, and contain a common 187 amino acid N-terminal domain. We show here that, unlike LAP2 β , LAP2 α behaved like a nuclear non-membrane protein in subcellular fractionation studies and was localized throughout the nuclear interior in interphase cells. It co-fractionated with LAP2 β in nuclear lamina/matrix-enriched fractions upon extraction of nuclei with detergent, salt and nucleases. During metaphase LAP2 α dissociated from chromosomes and became concentrated around the spindle poles. Furthermore, LAP2 α was mitotically phosphorylated, and phosphorylation correlated with increased LAP2 α solubility upon extraction of cells in physiological buffers. LAP2 α relocated to distinct sites around chromosomes at early stages of nuclear re-assembly and intermediately co-localized with peripheral lamin B and intranuclear lamin A structures at telophase. During *in vitro* nuclear assembly LAP2 α was dephosphorylated and assembled into insoluble chromatin-associated structures, and recombinant LAP2 α was found to interact with chromosomes *in vitro*. Some LAP2 α may also associate with membranes prior to chromatin attachment. Altogether the data suggest a role of LAP2 α in post-mitotic nuclear assembly and in the dynamic structural organization of the nucleus.

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Introduction

The nuclear envelope (NE) forms the boundary of the nucleus in eukaryotic cells and separates nuclear and cytoplasmic activities. It consists of a double membrane, an underlying filamentous meshwork (the nuclear lamina) and nuclear pore complexes which mediate nucleo-cytoplasmic transport (for reviews see Gerace and Burke, 1988; Moir *et al.*, 1995; Gant and Wilson, 1997). Although the outer and inner nuclear membranes are periodically joined at nuclear pore complexes, they are structurally and

functionally distinct. While the outer nuclear membrane is continuous with the smooth and rough endoplasmic reticulum, the inner nuclear membrane contains specific integral membrane proteins which link the membrane to the underlying lamina and to other nuclear components (reviewed in Georgatos *et al.*, 1994; Gerace and Foisner, 1994). The structural integrity of the NE is essential for nuclear functions such as DNA replication, RNA transcription and RNA processing. There is increasing evidence that the NE is involved in the organization of interphase chromatin, but the molecular mechanisms are not yet understood.

The lamins, intermediate filament-type proteins, comprising constitutively expressed B-type lamins and developmentally regulated A-type lamins, form a two-dimensional quasi-tetragonal network-like structure underneath the nuclear membrane and build the major structural framework of the NE (reviewed in Gerace and Burke, 1988). Previous studies, demonstrating *in vitro* interactions of lamins with chromosomes and chromatin (Glass and Gerace, 1990; Hoeger *et al.*, 1991; Yuan *et al.*, 1991; Glass *et al.*, 1993; Taniura *et al.*, 1995), the co-localization of lamins with DNA at the nuclear periphery (Paddy *et al.*, 1990; Belmont *et al.*, 1993; Fricker *et al.*, 1997) and within the nucleus (Bridger *et al.*, 1993; Moir *et al.*, 1994; Hozak *et al.*, 1995), and the lack of DNA replication in nuclei without an intact lamina (Newport *et al.*, 1990; Goldberg *et al.*, 1995; Ellis *et al.*, 1997; Spann *et al.*, 1997) suggested a role of lamins in chromatin organization. More recently, lamina-associated proteins have also been implicated in the structural organization of the nucleus. Several inner nuclear membrane proteins associated with the nuclear lamina have been characterized in higher eukaryotes: lamina-associated polypeptide (LAP) 1 (Senior and Gerace, 1988; Martin *et al.*, 1995); LAP2 (Foisner and Gerace, 1993; Furukawa *et al.*, 1995); p58/lamin B receptor (LBR) (Worman *et al.*, 1990); and otefin (Padan *et al.*, 1990). These proteins have been found to co-fractionate with detergent-high-salt-insoluble nuclear lamina/nuclear matrix preparations (Senior and Gerace, 1988; Worman *et al.*, 1988; Ashery-Padan *et al.*, 1997b) or to interact directly with lamins *in vitro* (Worman *et al.*, 1988; Foisner and Gerace, 1993). Several new compelling findings suggest that at least some of these proteins might also be directly involved in chromatin organization. p58/LBR and LAP2 have been shown to associate with chromosomes and chromatin *in vitro* (Foisner and Gerace, 1993; Pyrpasopoulou *et al.*, 1996), and might provide essential chromatin docking sites at the nuclear envelope. In a two-hybrid screen and by co-immunoprecipitation, LBR was found to interact with human chromodomain proteins homologous to *Drosophila* HP1, a heterochromatin protein involved in position effect variegation (Ye and Worman, 1996; Ye *et al.*, 1997).

A remarkable feature of the nucleus in higher eukaryotes is its ability to disassemble entirely at mitosis and then rapidly reassemble around daughter chromosomes (for recent reviews see Lourim and Krohne, 1994; Moir *et al.*, 1995; Foisner, 1997; Marshall and Wilson, 1997). NE breakdown, which involves nuclear membrane fragmentation and disassembly of the nuclear lamina, is triggered by mitosis-specific phosphorylation of the lamins (for review see Nigg, 1992), but there is evidence that the LAPs (Foisner and Gerace, 1993), LBR (Bailer *et al.*, 1991; Courvalin *et al.*, 1992; Nikolakaki *et al.*, 1997) and otefin (Ashery-Padan *et al.*, 1997a) are also subject to mitosis-specific phosphorylation and may contribute to mitotic nuclear disassembly. The post-mitotic assembly of nuclei, including the targeting of nuclear membranes to chromosomes, membrane fusion, nuclear pore complex assembly, lamina assembly and chromatin decondensation, offers an ideal system to study the potential role of lamins and associated proteins at different stages during the establishment of nuclear structure. The specific roles of lamins, LBR and LAPs in targeting nuclear membranes to the chromosomal surface at the end of mitosis has been controversial (reviewed in Lourim and Krohne, 1994; Foisner, 1997). Immunofluorescence microscopy has shown that LAP1, LAP2 (Foisner and Gerace, 1993; Maison *et al.*, 1997) and p58/LBR (Chaudhary and Courvalin, 1993; Ellenberg *et al.*, 1997) accumulate at the chromosomal surface in early stages of NE reformation in late anaphase before the bulk of lamins assemble into the lamina, suggesting that they may be involved in initial targeting of membranes to the chromosomes. Microinjection of recombinant proteins into mammalian cells revealed that fragments of LAP2 β which contain the lamin-binding domain inhibit nuclear volume increase and progression into S phase, but do not inhibit NE assembly (Yang *et al.*, 1997b).

The originally described LAP2 protein, first discovered in rat (Foisner and Gerace, 1993), has recently been shown to be a member of a family of related but distinct nuclear proteins derived from a single gene by alternative splicing (Harris *et al.*, 1994, 1995). Analysis of human LAP2 cDNAs and proteins, and of the human LAP2 gene, revealed three abundant forms: α (75 kDa), β (51 kDa) and γ (39 kDa). This analysis also revealed that an earlier proposed 49 amino acid secreted thymic polypeptide (named thymopoietin) was a proteolytic fragment from the N-terminus of nuclear LAP2 proteins, probably artificially generated during the isolation, and indicated that earlier claims of a physiological role for this fragment in neuromuscular transmission and T-cell function do not reflect the true cellular functions of the LAP2 proteins (Harris *et al.*, 1994, 1995). Following the α , β , γ nomenclature of Harris *et al.* (1994, 1995), LAP2 β , the human homologue of the originally described rat LAP2, and LAP2 γ both contain a putative transmembrane domain at their C-termini and are closely related structurally, the only difference being the insertion of a β -specific domain of 109 amino acids in LAP2 β . In contrast, the largest LAP2, LAP2 α , contains only a 187 amino acid N-terminal domain, identical to the N-termini of LAP2 β and LAP2 γ , which is followed by a 506 amino acid α -specific domain lacking a putative transmembrane region (Figure 1). Thus, LAP2 proteins are a family of related nuclear integral

membrane proteins as well as non-membrane associated proteins that may fulfil related functions in nuclear architecture and cell-cycle-dependent nuclear dynamics.

An additional nuclear membrane protein, possibly distantly related to LAP2 proteins, has recently been described. Emerin, the protein lost in patients with Emery–Dreyfuss muscular dystrophy (Bione *et al.*, 1994), is an ubiquitous Triton-resistant nuclear membrane protein (Manilal *et al.*, 1996; Nagano *et al.*, 1996) containing two regions with sequence similarity to LAP2: a 39 amino acid region near the N-terminus containing 16 identities and a C-terminal 34 amino acid region containing 14 identities that encompasses the membrane-spanning domain (Manilal *et al.*, 1996). Interestingly, the 187 residues common to all LAP2s includes the 38 amino acid region similar to the N-terminus of emerin. One possible explanation for the involvement of a nuclear membrane protein in a genetic muscle-wasting disease is that it may have a structural role in nuclear organization that affects muscle-specific functions.

Here, we present for the first time biochemical and structural data on the non-membrane bound LAP2 protein, LAP2 α . We show that the protein is associated with intranuclear structures in interphase and concentrates in areas of the mitotic spindle and in the midbody of mitotic cells. It relocates into the nucleus at early stages of post-mitotic nuclear assembly, giving rise to inter-chromosomal aggregates, intranuclear and peripheral structures, intermediately co-localizing with lamin A and lamin B, respectively. The redistribution of LAP2 α during cell division is correlated with a mitosis specific phosphorylation, and suggests a function of LAP2 α in the establishment of post-mitotic nuclear architecture.

Results

LAP2 α is associated with karyoskeletal components

To determine the subcellular distribution of LAP2 α we performed immunoblot analyses of nuclear and cytoplasmic fractions of normal rat kidney (NRK) cells using various monoclonal antibodies (mAbs) raised against common domains of the LAP2 proteins or against LAP2 α - and LAP2 β -specific regions. mAb 10, which was raised against a synthetic peptide corresponding to amino acids 29–50 within the common domain of LAP2 proteins (Figure 1), strongly recognized two proteins of ~75 and ~53 kDa exclusively in nuclei-enriched fractions of NRK cells (Figure 2). In contrast, mAb 15 which was generated against recombinant human LAP2 α , and mAb 11, generated against a synthetic peptide corresponding to α -specific amino acids 233–253, reacted only with the ~75 kDa protein in NRK nuclei and/or HeLa cell lysates (Figures 1 and 2). The ~53 kDa band detected by mAb 10 in NRK nuclei co-migrated with a HeLa protein immunoreactive with mAb 16 (Figure 2), an antibody raised against the β -specific amino acids 313–330 (Figure 1). Thus, it may be concluded that NRK and HeLa cells contain both LAP2 α and β , and that these proteins are predominantly nuclear.

Immunofluorescence microscopy of NRK cells using mAb 15 also revealed a predominantly nuclear localization of LAP2 α (Figure 3A). However, unlike LAP2 β (compare

Fig. 1. Schematic presentation of the domain organization of LAP2 isoforms and localization of mAb epitopes and cDNA constructs. The various domains of the LAP2 isoforms are indicated by different filling patterns. Black bars beneath the domains indicate regions known to contain epitopes for respective mAbs. Protein domains encoded by the respective pET constructs are indicated by thin filled bars. Numbers denote position of amino acids in the LAP2 sequences; arrows, minimal p34^{cdc2} phosphorylation consensus sites; and arrows plus asterisk, ideal p34^{cdc2} consensus sites. TM, transmembrane domain; NLS, nuclear localization signal.

with Foisner and Gerace, 1993; data not shown), LAP2 α was distributed throughout the nuclear interior being excluded only from the nucleoli. The nuclear localization of LAP2 α was clearly distinct from that of lamin B at the periphery of the NE, which gave rise to a nuclear rim staining (Figure 3A).

To investigate the intranuclear distribution of LAP2 α at the ultrastructural level, we performed immunoelectron microscopy of HeLa cells according to the Lowicryl low-temperature embedding protocol. This technique, which is clearly superior over conventional embedding protocols in terms of antigen preservation, gives a poorer structural preservation. Nevertheless, cells appeared to be intact and the nucleus was clearly discernible (Figure 4a). At higher magnification (Figure 4b) the inner nuclear membrane (IM) could nearly be traced along the entire nucleus, whereas the outer membrane (OM) and nuclear pore complexes (NP) were often disrupted due to a swelling of the luminal space. The LAP2 α -specific label on the Lowicryl sections was scattered over the entire nucleus and was sometimes closely associated with the inner membrane (Figure 4B, arrowheads), whereas the cytoplasm was completely free of label. Together, these data indicated that LAP2 α was located in intranuclear structures which were in close association with the NE at the nuclear periphery.

To test LAP2 α association with nuclear structures on a biochemical level, we extracted NRK nuclei in urea or non-ionic detergent and analyzed the distribution of both LAP2 proteins between soluble and insoluble fractions. Unlike LAP2 β , LAP2 α was solubilized in 7 M urea (Figure 5A), indicating that it is not inserted into the membrane bilayer. In contrast, LAP2 α and β behaved similarly upon extraction of nuclei with 1% Triton X-100 and increasing salt concentrations. At low ionic strength both proteins were completely retained in the low-speed pellet fraction (P_1), and also in more physiological ionic strength buffers (120 mM salt) the majority of the proteins remained insoluble (Figure 5A). Nuclease treatment of nuclei did not significantly change the solubility of the LAP2 proteins in Triton X-100-containing buffers. As the majority of the insoluble proteins sedimented at low-speed

Fig. 2. Identification of LAP2 proteins in NRK and HeLa cell fractions. NRK and HeLa cells were lysed in hypotonic buffer and total lysates (L), nuclei (N), and cytoplasmic (C) fractions were analyzed by SDS-PAGE and immunoblotting using mAbs as indicated. Coom, Coomassie Blue staining; numbers on the left, molecular weights $\times 10^{-3}$.

centrifugation, it may be concluded that both LAP2 α and β were associated with large cellular structures such as the nuclear lamina–nuclear matrix scaffold of the nuclei. At higher salt concentrations (250 mM) the majority of LAP2 α and β was soluble (Figure 5A), indicating that their association with the nuclear scaffold was weaker than previously reported for LAP2 and LAP1 (Senior and Gerace, 1988; Foisner and Gerace, 1993). Since these experiments were originally performed using fractions of rat liver NEs, we too tested the biochemical properties of LAP2 α in rat liver. An immunoreactive protein of ~75 kDa, co-migrating with NRK LAP2 α , was detected in ‘salt washed’ NE fractions isolated from rat liver nuclei but was absent from the post-nuclear microsomal membrane (MM) fraction (Figure 5B). Considering the harsh treatment of nuclei during the preparation of NEs, including extraction with DNase/RNase and with 0.5 M salt, the

Fig. 3. Cellular localization of LAP2 α . (A) NRK cells were processed for immunofluorescence microscopy using anti-LAP2 α mAb 15 and the DNA stain propidium iodide, or mAb 15 and a guinea pig antiserum to lamin B. (B) Five–10 μ m frozen sections of rat liver were processed for immunofluorescence microscopy including treatment with DNase and stained with mAb 10, directed against a common LAP2 epitope (LAP2) and propidium iodide (DNA), or with the LAP2 β -specific mAb 16 and propidium iodide. Confocal images are shown. Bars represent 10 μ m.

presence of LAP2 α in the NE fraction was unexpected and suggested that the protein was more tightly bound to karyoskeletal structures in rat liver than in NRK nuclei. Accordingly, the majority of rat liver LAP2 α was insoluble in 1% Triton X-100 plus 250 mM salt, while it was still efficiently solubilized in 7 M urea (Figure 5B). To test whether LAP2 α has the same subcellular distribution in rat liver tissue and in proliferating NRK cells, we performed immunofluorescence microscopy on cryosections of rat liver. mAb 10, directed against the common domain of LAP2 proteins, revealed staining throughout the nucleus and at the nuclear periphery (LAP; Figure 3B), while an antibody against the LAP2 β -specific domain stained the nuclear periphery exclusively. Therefore, the intranuclear staining detected by mAb 10 probably reflects the localization of the non-membrane-bound LAP2 isoform, LAP2 α . Unlike in NRK cells (Figure 3A), the LAP2 α -specific antibody mAb 15 did not detect the protein in rat liver nuclei using immunofluorescence microscopy (data not shown). The lack of staining might be caused by inaccessibility of the antigen due to a tight integration of LAP2 α in the nuclear structure, and is therefore consistent with the increased resistance of LAP2 α to detergent-salt extraction in rat liver tissue versus NRK cells.

LAP2 α localizes to different cytoplasmic and nuclear structures during mitosis

Nuclear structures are profoundly reorganized in the course of the cell cycle, involving the disassembly of the NE and the condensation of chromosomes at metaphase, and the post-mitotic re-establishment of nuclear architecture. We followed the cellular localization of LAP2 α at various cell-cycle stages by confocal immunofluorescence microscopy, using LAP2 α -specific mAbs. At prophase, when DNA starts to condense, LAP2 α was still located predominantly within the nucleus (Figure 6A). However, a superimposition of the LAP2 α (green) and the DNA (red) stain clearly showed that LAP2 α was mostly concentrated in the space between chromosomes, and showed only minor overlap (yellow) with DNA-containing structures. In metaphase and anaphase, when the NE was completely disassembled and DNA fully condensed, LAP2 α was found throughout the cell without an apparent association with the chromosomes. Instead, the protein seemed to be concentrated in areas of the mitotic spindle apparatus, particularly at the spindle pole regions (Figure 6B). At the initial stages of nuclear reassembly in early telophase, LAP2 α relocated into discrete structures between and around decondensing chromosomes, again mostly filling the space between DNA-containing structures (see superimposition in Figures 6C and D). At these cell-cycle stages the LAP2 α -specific staining in the cytoplasm was significantly weaker than that of the chromosome-associated LAP2 α structures. In contrast, both the lamin B- and lamin A-specific staining was still predominantly cytoplasmic (Figures 7A and B, upper panels). Thus, it can be concluded that LAP2 α associates with nuclear structures very early in nuclear reassembly, clearly before assembly of the bulk of lamins. Residual cytoplasmic staining of LAP2 α , however, was found in the midbody region of dividing cells (Figures 6D–F and 7). LAP2 α was detected at discrete spots and structures within the nuclei, and at the nuclear periphery as long as the DNA remained partially condensed (Figure 6D and E), and redistributed more or less uniformly throughout the nucleus after chromosome decondensation was complete (Figure 6F). The discrete LAP2 α structures at the nuclear periphery co-localized with lamin B, which was assembled into the nuclear lamina at late telophase/G₁ (Figure 7A, middle panels). Upon complete DNA decondensation, lamin B remained at the nuclear periphery, whereas LAP2 α redistributed into intranuclear structures (Figure 7A, lower panels). Therefore, LAP2 α may associate intermediately with the NE and lamin B at early stages of nuclear assembly. Lamin A redistributed to peripheral (data not shown) as well as intranuclear (Figure 7B, lower panels) structures in late telophase and G₁. As the intranuclear lamin A structures clearly overlapped with LAP2 α during G₁, LAP2 α might also associate with lamin-A-containing structures at specific cell-cycle stages and/or at specific nuclear sites.

LAP2 α is phosphorylated in a mitosis-specific manner

Since the phosphorylation of lamins, LAP2 β and other nuclear components has been suggested to regulate the mitotic reorganization of the proteins (Foisner, 1997), we tested whether LAP2 α was also modified in a mitosis-specific manner. NRK cells presynchronized by a double

Fig. 4. Ultrastructural localization of LAP2 α by immunoelectron microscopy. HeLa cells were processed for immunoelectron microscopy according to the low temperature Lowicryl embedding protocol and labelled with mAb 15. Low magnification image of a cell (**a**) and high magnification (**b**) of the area marked in **a** is shown. Arrowheads, LAP2 α -specific label at the nuclear membrane and within the nucleus; Nu, nucleus; IM, inner nuclear membrane; OM, outer nuclear membrane; NP, nuclear pore complex. Bars, 2.5 μ m in (**a**), 70 nm in (**b**).

Fig. 5. Solubilization of LAP2 proteins in various buffer conditions. (**A**) NRK nuclei were extracted with urea, Triton X-100, salt and DNase as indicated, and insoluble low-speed pellet fractions were collected by centrifugation at 2000 g for 10 min (P_1). The supernatant fraction was centrifuged at 100 000 g to yield high-speed pellet (P_2) and supernatant (S) fractions. Immunoblots of fractions using mAb 10 are shown. (**B**) Salt-washed NE fractions of rat liver (NE) were extracted with urea or Triton X-100 and salt, and insoluble (P) and soluble (S) fractions were collected by centrifugation at 15 000 g and analyzed by immunoblotting using mAb 15. MM, rat liver microsomal membrane fraction; NRK, total NRK cell lysate.

Fig. 6. Dynamic cellular organization of LAP2 α during the cell cycle. NRK cells were processed for immunofluorescence microscopy using LAP2 α -specific mAb 15 and the DNA stain propidium iodide. Single and superimposed confocal images of the LAP2 α and the DNA stain at various cell-cycle stages are shown. (A–F) show different stages of mitosis. Bars in A (for A and B) and in C (for C–F), 10 μ m.

thymidine block, or asynchronously growing HeLa cells were arrested at metaphase in nocodazole-containing medium, and mitotic cells were harvested by mechanical shake-off. FACS analyses (Figure 8B) and microscopy (not shown) revealed a mitotic index of >90% for these

mitotic cell fractions. Immunoblot analyses of mitotic and interphase cell extracts (Figure 8A, lanes M and I) or of LAP2 α immunoprecipitates from mitotic and interphase cells (data not shown), revealed a slightly reduced mobility of the mitotic LAP2 α in sodium dodecyl sulfate (SDS)

A **B**

Fig. 7. Co-localization of LAP2 α with lamins at early stages of NE assembly. NRK cells were processed for immunofluorescence microscopy using LAP2 α -specific mAb 15 and guinea pig antiserum to lamin B (**A**) or to lamin A (**B**). Single and superimposed confocal images of the LAP2 α and the lamin stain are shown. Note the co-localization of LAP2 α with lamin B at the periphery of newly formed nuclei, and of LAP2 α with lamin A in intranuclear structures. Bar, 10 μ m.

gels as compared with the interphase protein in both NRK and HeLa cells. To test whether the reduced mobility of mitotic LAP2 α might be caused by mitosis-specific phosphorylation, as has been reported for LAP2 β , we

analyzed mitotic and interphase LAP2 α immunoprecipitates by two-dimensional gel electrophoresis. Immunoblot analyses of isoelectric focusing gels showed that interphase LAP2 α focused at the basic end of the gel at a pI of $\sim$ 7.0

Fig. 8. LAP2 α is phosphorylated in a mitosis-specific manner. (A) Interphase (I) or nocodazole-arrested mitotic (M) NRK and HeLa cells [DNA FACS profiles are shown in (B)], were lysed, and cell lysates were analyzed by immunoblotting using mAb 15. (C) LAP2 α was immunoprecipitated from interphase (I) and mitotic (M) HeLa cell lysates, and single immunoprecipitates and mixtures of samples (I+M) were analyzed by two-dimensional gel electrophoresis and immunoblotting using mAb 15. Arrows denote the position of LAP2 α ; asterisks, immunoglobulin heavy chain; numbers and arrow on top, isoelectric points and isoelectric focusing gradient (IEF). (D) Mitotic NRK cell lysates were incubated for 0, 0.5 and 1 h at 30°C in the absence, or for 1 h in the presence of phosphatase inhibitors (IPI) and analyzed by immunoblotting.

(Figure 8C, panel I, small arrow), similar to immunoglobulin heavy chain (star). In contrast, mitotic LAP2 α immunoprecipitates focused in at least five distinct spots in a pI range of 5–6 (Figure 8C, panel M, small arrows), whereas the immunoglobulin heavy-chain as a control (star) was not shifted to acidic pI. Analysis of a mixture of interphase and mitotic samples (Figure 8C, panel I + M) clearly confirmed the differential position of the interphase versus mitotic LAP2 proteins. These results indicated that LAP2 α is more highly phosphorylated in mitosis than in interphase, and that at least five different phosphorylation sites might be targeted in a cell-cycle-dependent manner. In line with this observation, amino acid sequence analysis revealed two perfect and seven minimal consensus phosphorylation motifs for the mitotic p34^{cdc2} kinase in LAP2 α (Figure 1). The correlation between the phosphorylation state of LAP2 α and its mobility on SDS gels was further confirmed by incubating mitotic cell lysates at 30°C in the absence or presence of phosphatase inhibitors. As depicted in Figure 8D, incubation of lysates in the absence of phosphatase inhibitors for 1 h (lane M1) completely removed the molecular weight shift of mitotic LAP2 α in SDS gels, while incubation in the presence of

okadaic acid and calyculin for 1 h (M1PI) had no apparent effect on the protein's gel mobility.

LAP2 α is soluble during mitosis and reassembles *in vitro*

The different cellular localization of LAP2 α in interphase versus mitotic cells was also reflected by a different solubility of the protein in various buffer conditions. When S-phase cells were lysed in physiological ionic strength buffer (–) or in buffer plus 1% Triton X-100, the majority of LAP2 α was detected in the insoluble nuclear fractions (Figure 9A, Interphase). The protein was only solubilized at significantly higher ionic strength buffers (500 mM salt). In contrast, LAP2 α was completely soluble in mitotic cells under all conditions tested (Figure 9A, Metaphase). Thus, LAP2 α is apparently not associated with large cellular structures during mitosis, such as chromosomes, membrane vesicles or cytoskeletal networks. LAP2 β , in contrast, was recovered in the insoluble fraction upon lysis of mitotic cells in detergent-free buffers due to its integration in membrane vesicles, while it was mostly soluble in buffers containing 1% Triton X-100.

It has previously been demonstrated that NE assembly

Fig. 9. Different solubility of LAP2 α in interphase and mitotic cells and during *in vitro* nuclear assembly. **(A)** Interphase and metaphase cells were lysed in homogenization buffer alone (–) or in buffer containing 1% Triton, or 1% Triton and 500 mM salt, and insoluble (P) and soluble (S) fractions were obtained by centrifugation at 15 000 g. Immunoblots of fractions with LAP2 α -specific mAb 15 and with LAP2 β -specific mAb 16 are shown. **(B)** Lysates of mitotic NRK cells (0) were incubated for 0.5 or 1 h in the absence, or for 1 h in the presence of phosphatase inhibitors (1-PI) at 30°C. Insoluble (P) and soluble (S) fractions were obtained by centrifugation at 100 000 g with or without (–) prior addition of 1% Triton and 500 mM salt. Immunoblots using mAb 15 or antiserum to lamin B are shown. **(C)** Mitotic cell lysates were centrifuged at 15 000 g to remove chromosomes, and supernatant fractions were kept on ice (0) or incubated at 30°C for 1 h (1), and analyzed as above.

occurs *in vitro* upon incubation of mitotic cell lysates at 30°C (Burke and Gerace, 1986; Maison *et al.*, 1995). As shown in Figure 8D, incubation of mitotic cell lysates at 30°C for up to 1 h reverted the slightly decreased SDS gel mobility of mitotic LAP2 α to that of the interphase protein, suggesting that at least a partial exit from mitosis took place *in vitro* under these conditions. To test whether LAP2 α assembled into larger complexes during the incubation, cell lysates were centrifuged after various incubation times, and insoluble and soluble fractions were tested by immunoblot analyses. We found that the mitotic soluble LAP2 α increasingly shifted into the pellet fraction depending on the incubation time (Figure 9B). While after a 0.5 h incubation ~50% of the mitotic soluble protein was found in the pellet fraction, nearly all of the protein was converted into the insoluble form after 1 h incubation (Figure 9B). Control experiments, performed in the presence of phosphatase inhibitors revealed that the majority of the protein stayed soluble even after a 1 h incubation at 30°C (Figure 9B, 1-PI). Thus, the assembly of LAP2 α into larger structures was directly or indirectly dependent on dephosphorylation. Similarly, lamin B became increasingly insoluble upon incubation for 0.5 and 1 h in a dephosphorylation-dependent manner (Figure 9B). Both LAP2 α and lamin B remained mostly soluble in 1% Triton X-100 plus 500 mM salt at all stages of *in vitro* assembly,

indicating that unspecific aggregation of the protein due to partial denaturation did not occur in the extract.

To test whether the shift of LAP2 α from the soluble to insoluble fractions during incubation of mitotic lysates was dependent on the presence of chromosomes, we removed chromosomes by centrifugation prior to starting the *in vitro* assembly reaction. As depicted in Figure 9C, the protein distributed equally between soluble and insoluble fractions after a 1 h incubation of the chromosome-free mitotic cell fractions at 30°C. Therefore it can be concluded that LAP2 α may also partially assemble into larger protein complexes in the absence of chromosomes.

To study the kinetics of *in vitro* assembly in more detail, we performed differential centrifugation of whole-cell lysates after various incubation times and tested for the presence of histones, lamins and nuclear membrane proteins in subcellular fractions. Mitotic NRK cells were homogenized in the absence of phosphatase inhibitors, incubated at 30°C for up to 60 min and centrifuged at 2000 g to sediment large complexes which, according to the presence of histones in Coomassie Blue-stained samples, contained the majority of chromatin (Figure 10A, P₁). The supernatant fraction was then centrifuged at 100 000 g to sediment smaller structures, such as membrane vesicles (P₂). Immunoblot analyses of fractions obtained shortly after starting nuclear assembly by increas-

Fig. 10. Distribution of LAP2 α in chromosomal and membrane fractions during *in vitro* nuclear assembly. Mitotic NRK cells were homogenized in the absence of phosphatase inhibitors, and lysates were fractionated immediately (5 min) or after incubation for up to 60 min at 30°C. Interphase cells were lysed as controls. Lysates were centrifuged at 2000 *g* to yield a chromosome-enriched low speed pellet (P₁). Supernatants were centrifuged at 100 000 *g* to obtain membranes and large cytoskeletal structures in the high speed pellet (P₂) and soluble proteins in the supernatant (S). (A) Immunoblots of fractions using mAb 15 (LAP2 α), mAb RL 13 (LAP1), and an antiserum to lamins (Lamin). Coom, Coomassie Blue staining of fractions to detect histones. (B) Relative amounts of LAP2 α in S, P₁ and P₂ fractions revealed by densitometric scanning of immunoblots were plotted against incubation times.

ing the temperature to 30°C revealed that one half of the total cellular LAP2 α was found in the low-speed pellet, the other half in the soluble fraction (Figure 10A, 5 min). In contrast, lamins were still predominantly soluble, except for lamin B which was also found in the membrane-containing pellet fractions P₂ (Figure 10A, P₂), probably due to its reported membrane association during mitosis (Gerace and Burke, 1988). These observations confirmed our immunofluorescence data showing that LAP2 α assembled around chromosomes prior to the assembly of lamins (see above). As expected, the integral membrane protein LAP1 was detected in the membrane-containing high-speed pellet (P₂), but was absent from the soluble fraction (Figure 10A). In addition, large amounts of LAP1 were also found in the low-speed pellet fractions (P₁),

indicating that nuclear membranes were already attached to chromosomes at this early stage of nuclear reassembly.

Next, we followed the subcellular distribution of LAP2 α in the course of *in vitro* nuclear reassembly, and plotted the relative amounts of LAP2 α in supernatant and pellet fractions against the incubation time (Figure 10B). A rapid decrease of the soluble protein was detected within the first 30 min, accompanied by an increase of LAP2 α in the low-speed chromosome pellet (Figure 10B). Interestingly, we also detected an increase of the relative amount of LAP2 α in the high-speed, membrane-containing pellet fractions within the first 30 min, which declined again upon further incubation (Figure 10B, P₂). This result suggested that at least some of LAP2 α may associate with membranes before these membranes attach to chromosomes. Immunoblot analyses confirmed that the majority of LAP1 and lamins were also found in the chromosome fraction after 30 min incubation. In addition, these proteins were still present in the high-speed membrane pellet, indicating that they have not been completely incorporated into the NE and the karyoskeleton at this stage of nuclear assembly. By contrast, almost all of LAP2 α , as well as of LAP1 and lamins were found in the low-speed pellet in interphase cells.

LAP2 α interacts with chromosomes *in vitro*

To test whether LAP2 α can interact with chromosomes, we expressed the protein in *Escherichia coli* and performed *in vitro* co-sedimentation assays using metaphase chromosomes isolated from mitotic Chinese hamster ovary (CHO) cells. The soluble bacterial cell lysate contained a high concentration of recombinant protein of the expected molecular weight (Figure 11A), which was also detected by mAb 10 in immunoblot analysis (LAP2; Figure 11B). After incubation of chromosomes with bacterial cell lysates, chromosomes were sedimented through a sucrose cushion, and the pellet fractions were analyzed by immunoblotting using mAb 10. More than half of LAP2 α present in bacterial cell lysates was co-sedimented with chromosomes, whereas only a minor fraction of LAP2 α sedimented in the absence of chromosomes (Figure 11B). As chromosomes did not contain detectable amounts of immunoreactive endogenous LAP2 α and β (Figure 11B), it can be concluded that sedimented recombinant LAP2 α associated with components of the chromosomal fraction. Recombinant LAP2 β polypeptide, which contains the entire nucleoplasmic domain but lacks the transmembrane domain and the luminal C-terminus (Figure 1), was also efficiently co-sedimented with chromosomes. This confirmed previous results showing an *in vitro* interaction of purified LAP2 β with metaphase chromosomes (Foisner and Gerace, 1993). Immunofluorescence microscopy of chromosomes after incubation with bacterial cell lysates containing LAP2 α or LAP2 β revealed a uniform staining of the chromosomes with mAb 10. As this antibody did not stain chromosomes alone (data not shown), it can be concluded that both LAP2 α and β associated with the chromosomal surface and did not non-specifically aggregate with non-chromosomal structures. Considering that both proteins are identical in the first 187 N-terminal amino acids (Figure 1), one might speculate that this region is responsible for the proteins' interaction with chromosomes. To test this hypothesis, we performed co-

Fig. 11. *In vitro* interaction of recombinant LAP2 proteins with chromosomes. (A) LAP2 α 1-693, LAP2 1-187 and LAP2 β 1-408 were expressed in bacteria, and soluble cell fractions were analyzed by SDS-PAGE and Coomassie Blue staining. (B) Soluble cell fractions containing recombinant proteins (LAP2 +) or buffer (LAP2 -) were mixed with metaphase chromosomes (Chrom. +) or buffer (Chrom. -), sedimented through a sucrose cushion, and pellets were analyzed by immunoblotting using mAb 10. Lane LAP2, immunoblot of soluble bacterial cell lysates used for assay. Note that LAP2 β 1-408 is partially degraded, yielding two protein bands. (C) Incubation mixtures containing chromosomes and recombinant proteins were applied to adhesion slides and processed for immunofluorescence microscopy using mAb 10 and the DNA stain Hoechst. Bar represents 2 μ m.

sedimentation and immunofluorescence assays with a recombinant protein covering LAP2 amino acids 1-187 (Figure 11). However, an interaction of the N-terminal fragment with chromosomes was not found in either assay.

Discussion

Here we demonstrate the cell-cycle-dependent subcellular distribution and biochemical properties of a nuclear protein (LAP2 α) that has recently been identified as an alternatively spliced isoform of the inner nuclear membrane protein LAP2 (which we now call LAP2 β) (Harris *et al.*, 1994, 1995; Furukawa *et al.*, 1995). Unlike LAP2 β and several other LAP2 isoforms, LAP2 α lacks a putative transmembrane domain and was found throughout the nuclear interior, suggesting that the LAP2 proteins may be more generally involved in the establishment and maintenance of nuclear structure and not restricted to functions at the nuclear envelope. In addition to LAP2 α , LAP2 β and the β -related LAP2 γ , which together are the most abundant LAP2 proteins detected in a human T-cell line (Harris *et al.*, 1994), the possibility of additional

β -related proteins derived by alternative splicing, LAP2 δ , LAP2 ϵ , and LAP2 ζ , has recently been revealed by analysis of the mouse LAP2 gene and by re-analysis of the human LAP2 gene (Berger *et al.*, 1996; C.Harris, unpublished observation). Although LAP2 β has been shown to associate directly with lamins (Foisner and Gerace, 1993), and LAP2 α is shown here to co-localize with lamins at some cell-cycle stages, a direct interaction with the nuclear lamina has not been demonstrated for LAP2 α , or for LAP2 γ , δ , ϵ or ζ . One or more of the proteins may not associate directly with the lamina, and revision of the protein name may become appropriate as the molecular functions of each of the proteins become better understood.

LAP2 α —a karyoskeletal element?

A currently evolving view is that the nucleus contains a highly structured internal skeletal lattice that can organize chromosomes and numerous other nuclear components into physical and functional subdomains. Morphologically, this karyoskeletal fraction contains the nuclear lamina, attached nuclear pore complexes and in many instances a meshwork of filaments apparently derived from the nuclear

interior, poorly defined at the biochemical level. The karyoskeleton is broadly defined as the components that remain after nuclei are extracted with non-ionic detergents, nucleases and high salt. Applying these operational criteria, it is not entirely clear whether LAP2 α can be considered as a genuine component of the karyoskeleton in NRK nuclei. Although the majority of LAP2 α in NRK nuclei was found to remain insoluble in buffers containing 1% Triton X-100, physiological salt concentrations, and nucleases, it was almost completely soluble under high salt conditions (>250 mM). On the other hand, several observations are consistent with the LAP2 α function as a structural element of the karyoskeleton. First, upon extraction of NRK nuclei with Triton-containing buffers, LAP2 α behaved exactly like the membrane protein LAP2 β , which has previously been identified as a component of the nuclear lamina (Foisner and Gerace, 1993). Secondly, LAP2 α was present in NE fractions obtained from rat liver nuclei by extraction with nucleases and high salt (500 mM). Thirdly, unlike in NRK cells, LAP2 α -specific antibodies failed to detect the protein in rat liver nuclei by immunofluorescence microscopy, probably due to limitations of antigen accessibility. Thus, it is possible that the LAP2 proteins might be more tightly incorporated in the nucleoskeleton of differentiated non-proliferating cells in tissues as compared with the nucleoskeleton of highly proliferating cells in culture.

On the structural level, studies on the nucleoskeleton have always been difficult as it is normally concealed by a much larger mass of chromatin, and although several studies offer compelling evidence for the nucleoskeleton (Goldberg and Allen, 1992; Hozak *et al.*, 1995; Cordes *et al.*, 1997; Nickerson *et al.*, 1997), it is still uncertain whether the scaffold is one continuous element, several discrete elements or an *in vitro* artefact.

At the light- and electron-microscopic level, LAP2 α was found scattered throughout the nuclear interior with no obvious concentration in defined intranuclear structures being absent only from nucleoli. However, the relatively weak staining of interphase nuclei compared with mitotic cells, as well as the low density of LAP2 α -specific label in ultrathin Lowicryl sections in immunoelectron microscopy, indicated that only a fraction of the total cellular protein is detectable by the microscopic techniques in interphase cells, while the rest of the protein may be tightly integrated in higher chromatin structures, inaccessible for the antibodies. The masking of nuclear proteins during interphase, making them undetectable by conventional microscopic techniques, and the unmasking of the respective antigens to immunofluorescence staining upon entry of cells into mitosis has also been reported for other potential nuclear matrix proteins (Nickerson *et al.*, 1992).

Disassembly of LAP2 α structures during mitosis

According to our biochemical fractionation experiments LAP2 α became completely soluble during mitosis and was not apparently associated with large cellular structures, such as chromosomes or membrane vesicles. However, it remains unclear whether LAP2 α associates with other proteins during mitosis. The membrane-bound protein LAP2 β has also been shown to dissociate from chromosomes during mitosis, and the lack of an *in vitro* interaction of mitotically phosphorylated LAP2 β with lamin B

(Foisner and Gerace, 1993) suggested that LAP2 β did not interact with lamins during mitosis. This is in contrast to other lamina-associated proteins of the inner membrane, such as LBR and LAP1, which have been suggested to exist in multimeric complexes with lamins and possibly other proteins during mitosis (Meier and Georgatos, 1994; Maison *et al.*, 1997).

Although at the biochemical level we did not detect any interaction of LAP2 α with cytoskeletal proteins in mitotic cells, immunofluorescence microscopy showed a concentration of LAP2 α in the areas of the mitotic spindle apparatus, particularly at spindle poles. The biological significance of this is not clear, but similar distributions have been reported for other nuclear proteins such as LAP1 (Maison *et al.*, 1997), NuMA (for review see Compton and Cleveland, 1994), protein 4.1 (Krauss *et al.*, 1997) and a structural nuclear matrix antigen (Nickerson *et al.*, 1992). The recently reported association of LAP1 with the mitotic spindle apparatus (Maison *et al.*, 1997) has been suggested to play a role in membrane partitioning during cell division, separating a subset of nuclear membrane vesicles from other nuclear and endoplasmic reticulum-derived vesicles. However, these data are controversial as more recent studies have demonstrated the diffusion and distribution of nuclear membrane proteins throughout the cytoplasmic membrane system at the onset of mitosis (Ellenberg *et al.*, 1997; Yang *et al.*, 1997a).

NuMA has been found to play an integral role in the establishment and maintenance of nuclear structure by antibody microinjection (Kallajoki *et al.*, 1993) and NuMA transfection (Compton and Cleveland, 1993) experiments, and it has recently been shown to play a crucial role in the assembly of the mitotic spindle itself (Gaglio *et al.*, 1995; Merdes *et al.*, 1996). In addition, as NuMA associates with microtubules during metaphase and progressively accumulates at the polar ends of the mitotic spindle, when cells proceed to anaphase, it has been suggested that the association of NuMA and possibly other nuclear proteins with the mitotic spindle apparatus may provide a mechanism to distribute the proteins equally between daughter cells. This mechanism of post-mitotic nuclear accumulation is distinct from the passive diffusion and active nuclear transport of most other nuclear proteins, including lamins, and might be particularly important for proteins that are essential for early stages of nuclear reassembly and that associate with chromosomes prior to nuclear membrane formation. Therefore, considering the potential role of LAP2 α in early stages of nuclear reassembly (see below), its association with the mitotic spindle may ensure an efficient interaction of LAP2 α with chromosomes at the end of mitosis. It remains unclear whether LAP2 α can directly interact with microtubules. Based on our observations that a significant fraction of the protein associated with the mitotic spindle and that some of the protein ended up in the midbody, it might be speculated that LAP2 α may transiently gain microtubule binding activity, probably similar to the previously reported chromosome passenger proteins (Earnshaw and Bernat, 1990).

As for the regulation of LAP2 α relocalization, it is very likely that, similar to lamins, LAPs and LBR (reviewed in Foisner, 1997), mitosis-specific phosphorylation is one of the key mechanisms. Although the effect of phosphoryl-

ation on LAP2 α dynamics has not yet been shown directly, a number of observations argue for a phosphorylation-dependent mechanism. There is a perfect temporal correlation of phosphorylation and solubilization of the protein in both our subcellular fractionation and our *in vitro* assembly studies. Furthermore, the dephosphorylation and the assembly of LAP2 α into insoluble structures in our *in vitro* nuclear assembly studies were inhibited by phosphatase inhibitors. Two-dimensional gel electrophoresis revealed at least five mitosis-specific spots, suggesting that a minimum of five potential phosphorylation sites are targeted during mitosis. It remains to be analyzed whether these differentially phosphorylated LAP2 α proteins exist simultaneously in the mitotic cell or whether specific sites are phosphorylated at different stages of mitosis. Alternatively, a partial dephosphorylation might occur during cell lysis and sample preparation. Nevertheless, the presence of various consensus sites for p34^{cdc2}-dependent phosphorylation in the primary sequence of the polypeptide (Figure 1) is consistent with a mitosis-specific phosphorylation of the protein.

Potential role of LAP2 α in nuclear assembly

The assembly of LAP2 α structures at distinct sites around chromosomes during early stages of post-mitotic nuclear reassembly, when the majority of lamins was not yet assembled, suggests a role for LAP2 α in the establishment of nuclear structure. Its inter-chromosomal localization would be consistent with a function of LAP2 α in tethering together the telophase bundle of chromosomes. It is unclear whether LAP2 α associates with chromosomes prior to the formation of a closed nucleus, or whether LAP2 α , which contains a putative nuclear localization sequence (Figure 1), enters the nucleus by active transport through newly formed pore complexes. The early association of LAP2 α with chromosomes and its nuclear accumulation before lamina assembly, as well as the concentration of LAP2 α at the spindle poles in close vicinity to the separated sets of chromosomes, argue for a direct access of LAP2 α to chromosomal structures prior to nuclear membrane formation at least at this cell cycle stage. The demonstrated *in vitro* interaction of LAP2 α with isolated chromosomes is also consistent with this model. Nuclear localization signal (NLS)-mediated nuclear transport of LAP2 α might be relevant for newly synthesized protein during interphase, although the functional significance of the NLS in LAP2 α has not yet been shown directly.

The nature of the molecular interactions between LAP2 α and chromosomes remains to be solved. Neither the chromosomal components interacting with LAP2 α , nor the molecular domains of LAP2 α involved in the interaction have yet been identified. Our observation that both recombinant LAP2 α and the nucleoplasmic domain of LAP2 β interacted with chromosomes *in vitro* would suggest that the chromosome interaction site of the protein is located within their identical N-termini, consistent with a recently reported chromatin binding site in the N-terminal 85 amino acids of LAP2 β (Yang *et al.*, 1997b; Furukawa *et al.*, 1998). However, the N-terminal LAP2 fragment (amino acids 1–187) alone failed to interact with chromosomes in our assays. There are several explanations for this apparent inconsistency. The recombinant bacterially expressed protein may lack certain post-translational modi-

fications or may be misfolded, and thus unable to interact with chromosomes. Alternatively, LAP2 α and LAP2 β may contain additional chromosome-binding sites in their C-terminal regions. In line with this hypothesis, the interaction of LAP2 β with chromosomes has recently been suggested to involve multiple domains of the protein, located in the β -specific region (Furukawa *et al.*, 1997).

Interestingly, we found a co-localization of LAP2 α with lamin B at discrete structures at the nuclear periphery and with lamin A in intranuclear structures in late anaphase and G₁ phase. This might indicate a direct or indirect association of at least some of the cellular LAP2 α with lamins at specific cell-cycle stages. The close association of a subset of LAP2 α -specific label with the NE in immunoelectron microscopy and the association of LAP2 α with lamin-containing cell fractions in the *in vitro* nuclear assembly studies and in subcellular fractionation experiments are consistent with a LAP2 α -lamin interaction. Furthermore, we observed an *in vitro* interaction of recombinant LAP2 α with purified lamins (our unpublished results).

Overall, our data indicate that LAP2 α is a nuclear protein that is involved in the structural organization of the nucleus and in the post-mitotic nuclear assembly. Functional studies involving the inducible expression of mutated LAP2 proteins in mammalian cells are in progress to unravel the specific function(s) of LAP2 α in the various steps of nuclear assembly, involving membrane targeting, chromosome decondensation, lamin assembly and nuclear growth.

Materials and methods

Cell culture and synchronization

NRK, CHO and HeLa cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50 μ g/ml of penicillin and streptomycin (all from Life Technologies, Paisley, UK) at 37°C in a humidified atmosphere containing 5% CO₂. For synchronization of cell growth, NRK and CHO cell cultures were arrested in G₁/S phase by an overnight incubation in complete DMEM or in Joklik's modified minimum essential medium (S-MEM, Life Technologies) with 10% FCS, a non-essential amino acid mix and penicillin, streptomycin, plus 2 mM thymidine. In some cases a second overnight incubation in thymidine-containing medium was applied following a 10–12 h release from the block in complete medium without thymidine. To select S-phase cells, cultures were released from the thymidine block for 4 h. For the collection of mitotic cells, cultures were released from the thymidine block for 4 h and cultivated for an additional 4–6 h in growth medium containing 0.2 μ g/ml nocodazole (Calbiochem-Behring Corp., La Jolla, CA). Weakly attached mitotic cells were harvested by mechanical shake off. HeLa cells were incubated for 16–24 h in medium containing 0.2 μ g/ml nocodazole without preincubation in thymidine-containing medium. For FACS analyses, $\sim 5 \times 10^5$ cells in 1 ml phosphate-buffered saline (PBS) were fixed in 4 ml of 85% ethanol at –20°C, and cell pellets were briefly treated with 0.05% aqueous pepsin, stained with 2 μ g/ml 4,6-diamidino-2-phenylindole and 15 μ g/ml sulforhodamine (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany), in 100 mM Tris-HCl pH 8.0, 2 mM MgCl₂, 0.1% Triton X-100, and analyzed with a Partec PAS II flow cytometer (Partec, Münster, Germany).

Cell fractionation and *in vitro* assembly

Cells were incubated in ~ 5 vol. ice-cold hypotonic buffer (10 mM HEPES pH 7.4, 5 mM MgCl₂, 10 mM NaCl, 1 mM DTT) containing the protease inhibitors PMSF (1 mM), benzamidine (2 mM), and aprotinin, leupeptin, and pepstatin (2 μ g/ml each), and homogenized in a glass–glass homogenizer by 100 strokes with a tight pestle. After addition of 8% sucrose, the soluble cytoplasmic and the insoluble nuclear fractions were separated by centrifugation at 2000 g for 20 min at 4°C.

The nuclei-containing pellets were extracted in the same buffer containing either 1% Triton X-100 and 50–250 mM NaCl or 7 M urea, and centrifuged at 2000 or 100 000 *g* to yield a low-speed and a high-speed pellet, and a high-speed supernatant fraction.

For analyzing solubility of proteins in interphase versus mitotic cells, synchronized cell cultures were homogenized in ice-cold homogenization buffer (50 mM HEPES pH 7.4, 4 mM MgCl₂, 10 mM EGTA, 100 mM NaCl, 0.1 mM DTT) containing protease inhibitors, 20 µM cytochalasin B (Sigma-Aldrich Chemie GmbH), phosphatase inhibitors (0.1 µM calyculin, 8 µM microcystin, 0.1 µM okadaic acid, 1 mM Na-pyrophosphate, 0.5 mM β-glycerophosphate) and kinase inhibitors (0.1 µM olomoucine, 0.5 µg/ml staurosporine, 0.5 mM H7) (Sigma-Aldrich Chemie GmbH, Life Technologies, and Calbiochem-Behring Corp.). Samples were centrifuged at 15 000 *g* for 10 min without prior treatment, or after the addition of 1% Triton X-100 and/or 500 mM NaCl, and/or 500 µg/ml DNase and 200 µg/ml RNase (Boehringer Mannheim, Germany).

For *in vitro* nuclear assembly, mitotic NRK or HeLa cells were homogenized in homogenization buffer without phosphatase and kinase inhibitors, and cell lysates were incubated for up to 2 h at 30°C. Phosphatase and kinase inhibitors were then added and samples were centrifuged at 2000 or 100 000 *g* for 20 min with or without prior addition of 1% Triton X-100 and/or 500 mM salt. In some cases, *in vitro* assembly was performed in KHM buffer as described previously (Burke and Gerace, 1986). To test the assembly of LAP2α in the absence of chromosomes mitotic cell lysates were centrifuged for 10 min at 15 000 *g* and supernatants were used in the assembly reaction.

NEs and microsomal membranes were isolated from rat liver, and 'salt washed' and extracted with urea, Triton X-100 and various salt concentrations as described previously (Foisner and Gerace, 1993).

Isolation of metaphase chromosomes

Nocodazole-arrested CHO cells obtained from ten roller bottles were incubated in complete medium containing 20 µM cytochalasin B and 0.2 µg/ml nocodazole for 30 min at 37°C, preswollen in 5 mM HEPES pH 7.4, 5 mM NaCl, 5 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT and protease inhibitors (chromosome buffer) for 10 min on ice, and homogenized in the same buffer containing 0.1% Triton X-100. The cell lysate was layered on top of a discontinuous sucrose gradient (3 ml of 20, 30, 40, 50 and 60% sucrose in chromosome buffer), centrifuged for 10 min at 1500 r.p.m. (Megafuge 1.0R, Heraeus Sepatech GmbH, Osterode, Germany) at 4°C, and chromosomes enriched in a diffuse band in the middle of the gradient were collected.

Expression and isolation of recombinant LAP proteins

The construction of plasmids pET17b-LAP2α (formerly called pET 17b-hTPO) is described elsewhere (Harris *et al.*, 1994); pET23a-LAP2β 1–408 and pET23a-LAP2 1–187 were constructed by PCR amplification of sequences encoding LAP2β amino acids 1–408 and LAP2αβγ amino acids 1–187 (Harris *et al.*, 1994), and insertion into pET 23a (Novagen Inc., Madison, WI) via *Nde*I and *Xho*I restriction sites. Recombinant proteins were expressed in *E.coli* BL21 (DE3) using the inducible T7 RNA polymerase-dependent pET vector system (Novagen). Protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) for 2–4 h, and bacteria were harvested by centrifugation at 4000 r.p.m. for 5 min (Heraeus Megafuge 1.0R). Bacteria were frozen in 1/10 of the original culture volume of Tris buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 2 mM EGTA, 1 mM DTT, protease inhibitors), thawed, and lysed by addition of 0.1 mg/ml lysozyme, 0.1% Triton X-100, 10 mM MgCl₂, 50 µg/ml DNase, and 20 µg/ml RNase and incubation for 30 min at 30°C. Following the addition of 7 M urea and homogenization in a glass–glass homogenizer, cell lysates were spun at 4000 r.p.m. (Heraeus Megafuge) for 10 min and subsequently at 35 000 r.p.m. (TLA 45 rotor, Beckmann Instruments Inc., Palo Alto, CA) for 30 min and supernatants were stored at –20°C.

Chromosome binding assays

Soluble bacterial lysate fractions were dialyzed against binding buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 2 mM EGTA, 0.2% Triton X-100, 1 mM DTT and protease inhibitors), incubated for 30 min at 30°C and centrifuged in an Eppendorf microfuge for 30 min at 13 000 *g*. The supernatants were analyzed by SDS–PAGE and diluted with binding buffer to obtain similar concentrations of recombinant proteins. An aliquot (300 µl) of the soluble fraction was mixed with 150 µl of chromosome buffer or chromosomes, incubated for 30 min at 30°C and centrifuged through 100 µl of a 35% sucrose cushion in binding buffer for 10 min at 4000 r.p.m. (Heraeus Megafuge 1.0R). Pellet fractions were analyzed by SDS–PAGE and immunoblotting.

For detection of bound protein on individual chromosomes, 30 µl of the chromosome–protein mixture was placed on adhesion slides (Bio-Rad, Hercules, CA), and attached complexes were analyzed by immunofluorescence.

Immunofluorescence and immunoelectron microscopy

Cells grown on plastic Petri dishes or chromosomes attached to adhesion slides were fixed with 3.7% formaldehyde in PBS for 20 min at room temperature with or without prior permeabilization in PBS plus 0.1% Triton X-100 for 5 min. After incubation in 50 mM NH₄Cl in PBS and permeabilization of the fixed cells in PBS/0.1% Triton X-100 for 5 min each, samples were incubated in PBS plus 0.2% gelatine for 30 min. Primary and secondary antibodies were applied in PBS plus gelatine for 1 h each at room temperature. Primary antibodies used were hybridoma supernatants (undiluted), purified LAP2 antibodies (5–10 µg/ml) (Harris *et al.*, 1994), affinity-purified guinea pig anti-lamin B antibodies (10 µg/ml), and guinea pig anti lamin A/C antibody (1:100) (Ottaviano and Gerace, 1985; Foisner and Gerace, 1993), secondary antibodies, goat anti-mouse IgG conjugated to Bodipy FL (Molecular Probes, Leiden NL), and goat anti-guinea pig IgG conjugated to Texas Red (Accurate Chemicals & Scientific Corp., Westbury, NY). After several washes in PBS, DNA was stained with 1 µg/ml Hoechst dye 33258 (Calbiochem-Behring Corp.) for 10 min, or RNA was digested with 10 µg/ml DNase free RNase for 30 min at room temperature, and DNA was stained in 0.1 µg/ml propidium iodide (Sigma-Aldrich Chemie GmbH) in PBS for 10 min. Samples were mounted in Mowiol and viewed in a Zeiss Axiophot microscope and a MRC-600 confocal microscope (Bio-Rad).

For immunolocalization of LAP2 isoforms in rat liver tissue, frozen tissue sections (5–10 µm) cut on a cryotome were fixed and stained as described above, except that samples were treated with 10 µg/ml DNase for 30 min after fixation, 0.2% gelatine, 1% bovine serum albumin (BSA), and goat serum (1:50 in PBS) were used for blocking, and secondary antibodies were preabsorbed on rat liver acetone powder.

For immunoelectron microscopy, HeLa cells were fixed for 1 h in 4% paraformaldehyde, suspended in 10% gelatine at 37°C and chilled on ice. Small cubes of the samples were dehydrated in ethanol at progressively lower temperatures, embedded in Lowicryl K4M (Agar Scientific Ltd, Stansted, UK), and polymerized at –35°C by UV light (Carlemalm *et al.*, 1982). Ultrathin sections were cut on a Reichert Ultratrac S ultramicrotome (Reichert Division Leica AG, Vienna, Austria), and processed for immunolabeling using 2% BSA plus 0.2% gelatine in PBS as blocking reagent, anti-LAP2α mAb 15 at 10 µg/ml, and goat anti-mouse IgG coupled to 10 nm gold particles (British BioCell International, Cardiff, UK; diluted 1:20). Sections were stained with uranyl acetate and lead citrate, and viewed in a JEOL 1210 transmission electron microscope at 80 kV.

Immunoprecipitation

Mitotic or interphase HeLa cells were lysed in homogenization buffer containing 1% Triton X-100, 500 µg/ml DNase, 200 µg/ml RNase, and protease-, kinase- and phosphatase inhibitors. Following a 10 min incubation at room temperature, 1% SDS was added to the samples. After 10-fold dilution with 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100 and 5 mM EDTA the samples were centrifuged for 10 min in an Eppendorf centrifuge, and incubated with 100 µl 10% (w/v) protein A–Sepharose (Pharmacia Biotech, Uppsala, Sweden). Following removal of the beads by centrifugation in an Eppendorf microfuge, supernatants (1 ml) were incubated with 5–10 µg mAb 15 by end-over-end rotation for 15–20 h at 4°C. After addition of 100 µl protein G–Sepharose beads (Sigma-Aldrich Chemie GmbH) samples were incubated for an additional 2 h, and beads were pelleted through 30% sucrose at 1000 r.p.m. (Heraeus Megafuge 1.0R) for 2 min at 4°C, washed with buffer and prepared for two-dimensional gel electrophoresis.

Gel electrophoresis and immunoblotting

One-dimensional SDS–PAGE was performed according to Laemmli (1970). Two-dimensional gel electrophoresis was performed as previously described (Gotzmann *et al.*, 1997). For immunoblotting, proteins separated on one- or two-dimensional gels were electrophoretically transferred onto nitrocellulose (0.2 µm Schleicher and Schuell, Inc., Dassel Germany) in 48 mM Tris-HCl pH 9.4, 39 mM glycine using the Mini Transblot system (Bio-Rad). For the immunological detection of proteins the Protoblot Immunoscreening System (Promega, Madison, WI) or the SuperSignal detection system (Pierce, Rockford, IL) were used. Primary antibodies used were anti-LAP2 antibodies (hybridoma supernatants, undiluted), anti-LAP1 (Senior and Gerace, 1988; RL13 ascites fluid, diluted 1:1000), antiserum to lamin B (Foisner *et al.*, 1991; diluted

1:1000) and antiserum to lamins A/C (Glass *et al.*, 1993; diluted 1:1000). Quantification of stained protein bands was done using the NIH image software.

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