Lamin-binding Fragment of LAP2 Inhibits Increase in Nuclear Volume during the Cell Cycle and Progression into S Phase

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Abstract. Lamina-associated polypeptide 2 (LAP2) is an integral membrane protein of the inner nuclear membrane that binds to both lamin B and chromatin and has a putative role in nuclear envelope (NE) organization. We found that microinjection of a recombinant polypeptide comprising the nucleoplasmic domain of rat LAP2 (residues 1–398) into metaphase HeLa cells does not affect the reassembly of transport-competent nuclei containing NEs and lamina, but strongly inhibits nuclear volume increase. This effect appears to be specifically due to lamin binding, because it also is caused by microinjection of the minimal lamin-binding region of LAP2 (residues 298–373) but not by the chromatin-binding domain (residues 1–88). Injection of the lamin-binding region of rat LAP2 into early G1 phase HeLa cells also strongly affects nuclear growth; it almost completely prevents the threefold nuclear volume increase that normally occurs during the ensuing 10 h. Moreover, injection of the fragment during early G1 phase strongly inhibits entry of cells into S phase, whereas injection during S phase has no apparent effect on ongoing DNA replication. Since the lamin-binding fragment of LAP2 most likely acts by inhibiting dynamics of the nuclear lamina, our results suggest that a normal function of LAP2 involves regulation of nuclear lamina growth. These data also suggest that lamina dynamics are required for growth of the NE and for nuclear volume increase during the cell cycle, and that progression into S phase is dependent on the acquisition of a certain nuclear volume.

THE nuclear lamina, which is a filamentous protein meshwork lining the inner nuclear membrane, is thought to provide a structural framework for the nuclear envelope (NE)[1] and an anchoring site for chromatin at the nuclear periphery (for review see Nigg, 1992; Georgatos et al., 1994). The lamina consists of a polymeric assembly of intermediate-type filament proteins (nuclear lamins) and a number of more minor lamina-associated polypeptides (LAPs). Four major lamin isotypes (A, B_1, B_2, and C) are present in mammalian cells (for review see Nigg, 1992). B lamins are expressed throughout development, whereas A lamins appear at about the time of terminal differentiation, or after it. Three lamina-associated polypeptides have been characterized in detail in mammalian cells: LAP1, LAP2, and a protein called the lamin B receptor (LBR) (for review see Gerace and Foisner, 1994). All three polypeptides are integral membrane proteins that directly bind to lamins. LAP2 (Foisner and Gerace, 1993) and LBR (Worman et al., 1988) preferentially interact with lamin B, whereas LAP1 (Foisner and Gerace, 1993) interacts with both A and B lamin isotypes. LAP1 (Martin et al., 1995) and LAP2 (Furukawa et al., 1995) each have a large nucleoplasmic domain and a single predicted membrane-spanning segment, whereas LBR (Worman et al., 1990) has eight predicted membrane-spanning segments and contains a large region with homology to sterol C14 reductase of *Saccharomyces cerevisiae* (see Georgatos et al., 1994). The primary transcript of the LAP2 gene is alternatively spliced (Harris et al., 1994, 1995; Berger et al., 1996) and gives rise to at least three different proteins: thymopoietins α, β (LAP2), and γ (Harris et al., 1995). Thymopoietin α is identical to LAP2 only for the first 187 amino acids, and lacks an apparent membrane-spanning domain; whereas thymopoietin γ contains a deletion of 109 amino acids in the equivalent of the nucleoplasmic domain of LAP2 (Harris et al., 1995).

It is likely that the attachment of the nuclear lamina to the inner nuclear membrane is due, at least in part, to the association of lamins with certain integral membrane proteins of the inner nuclear membrane (Gerace and Foisner, 1994). Lipid modification (farnesylation) of B lamins (Nigg, 1992) also may be important for nuclear membrane attachment. Integral proteins of the inner nuclear mem-

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1. *Abbreviations used in this paper*: BrdU, bromodeoxyuridine; GST, glutathione-S-transferase; LAP, lamina-associated polypeptide; LBR, lamin B receptor; MBP, maltose-binding protein; NE, nuclear envelope; NPC, nuclear pore complex.

brane also could have a role in modulating the structure of lamin filaments (e.g., filament width) and/or in regulating the growth of the lamina that occurs during interphase in cycling cells (Gerace and Foisner, 1994). However, which integral proteins are involved in lamina binding to the inner nuclear membrane or in lamina structure has not been determined by functional studies.

The association of the nuclear lamina with chromatin is suggested to involve both nuclear lamins and lamina-associated polypeptides. In vitro binding studies show that lamins can interact with various chromatin substrates (Burke, 1990; Glass and Gerace, 1990; Hoger et al., 1991; Yuan et al., 1991; Taniura et al., 1995). Quantitative binding analysis has shown that the COOH-terminal tail domains of lamins directly associate with isolated rat liver chromatin with apparent K_ds of ~100–300 nM, and interact specifically with core histones (Taniura et al., 1995). Purified LAP2 associates with chromosomes isolated from mitotic cells, and its binding to both lamins and chromosomes is negatively regulated by mitotic LAP2 phosphorylation (Foisner and Gerace, 1993). LBR, which interacts with the heterochromatin protein HP1 (Ye and Worman, 1996), has been suggested to be an additional chromatin-binding component of the lamina (Pyrpasopoulou et al., 1996; Ye and Worman, 1996). LBR undergoes mitosis-specific phosphorylation (Courvalin et al., 1992; Nikolakaki et al., 1997), but whether this affects HP1 or lamin binding is unknown. The chromatin-binding properties of these lamina components could reflect an involvement in a number of possible functions: reassembly of the NE at the end of mitosis (see below), anchoring of higher order chromatin domains to the NE during interphase, and regulation of specific interphase chromatin activities such as DNA replication (Gerace and Foisner, 1994).

There are several ways in which the lamina might be linked to regulation of NE structure during mitosis and interphase. During mitosis in higher eukaryotic cells, the NE is disassembled and lamins are depolymerized (for review see Nigg, 1992). Concomitantly, integral membrane proteins of the NE become dispersed throughout ER membranes (Yang et al., 1997). Disassembly of the lamina is likely to be a prerequisite for nuclear membrane disassembly. The reassembly of nuclear membranes around chromatin that takes place in late anaphase is accompanied by the accumulation of LAPs, LBR, and lamins at the chromosome surfaces (for review see Gerace and Foisner, 1994; Marshall and Wilson, 1997). This process is hypothesized to be driven by cooperative binding interactions between integral membrane proteins, chromatin components, and lamins (Yang et al., 1997). Because of its properties as an intermediate filament meshwork, the lamina could contribute mechanical stability to the NE during interphase, and could also play an important role in regulating the interphase growth of the NE that occurs in cycling cells (Gerace and Burke, 1988). A role for the lamina in nuclear growth is consistent with studies involving the assembly of lamin-deficient nuclei in vivo in mitotic mammalian cells (Benavente and Krohne, 1986) and in vitro in *Xenopus laevis* egg extracts (Newport et al., 1990; Meier et al., 1991; Goldberg et al., 1995; Spann et al., 1997), where nuclear size increase was impaired upon lamin depletion.

In this study, we have analyzed the functions of LAP2 by microinjecting recombinant fragments of this protein into HeLa cells. We found that injection of a 398–amino acid fragment comprising the nucleoplasmic domain of LAP2 into metaphase cells had no detectable effect on subsequent reassembly of the NE, but strongly inhibited the postmitotic increase in nuclear volume. This effect was most likely because of lamin binding, because it also was obtained with a 76–amino acid fragment of LAP2 comprising its lamin-binding region. Similarly, injection of the lamin-binding fragment of LAP2 into early G1 phase cells almost completely inhibited the ~threefold increase in nuclear volume that normally occurred in the subsequent 10 h, and correspondingly inhibited entry of cells into S phase. These data suggest a function for LAP2 in regulating dynamics of the nuclear lamina, and support the possibility that the nuclear lamina is directly involved in nuclear volume increase during interphase of cycling cells. We discuss the possible existence of a cell cycle checkpoint linking S phase to nuclear volume.

Materials and Methods

Expression of Recombinant LAP2 Fragments

For production of the glutathione-S-transferase (GST) fusion protein containing amino acids 298–373 of LAP2, the corresponding cDNA sequence was excised from the rat LAP2 cDNA clone (Furukawa et al., 1995) by digestion with HincII and XhoI, and then was inserted into SmaI and XhoI sites of the vector pGEX-2T (Pharmacia Biotechnology, Inc., Piscataway, NJ). The resulting plasmid was transformed into *Escherichia coli* strain BL21. The GST fusion protein was expressed and purified according to the manufacturer's protocol.

To obtain an expression plasmid encoding the maltose-binding protein (MBP) fused to residues 298–373 of LAP2, the cDNA sequence encoding the corresponding region of LAP2 was excised by BamHI and HindIII digestion from the vector pGEX-LAP2 (298–373), and then was inserted into the vector pMALc (New England Biolabs, Inc., Beverly, MA). A DNA linker was prepared by hybridizing two oligonucleotides (5′-AGCTTAGTCTAGACTAG-3′ and 5′-AGCTCTAGTCTAGACTA-3′) and inserting this into the HindIII site of the previous pMALc-LAP2 (298–373) construct to create a termination codon for the fusion protein. To obtain an expression plasmid encoding MBP fused to residues 1–88 of LAP2, the relevant LAP2 sequence was cleaved by BamHI and HindIII digestion from plasmid $(His)_6$-LAP2 (1–88) (provided by Dr. C. Fritze, The Scripps Research Institute, La Jolla, CA), and then was inserted into the pMALc vector containing the linker with the termination codon.

MBP-LAP2 (298–373) and MBP-LAP2 (1–88) were expressed in *E. coli* strain BL21 and were affinity purified by binding to amylase beads according to the manufacturer's instructions (New England Biolabs, Inc.). The MBP-LAP2 (1–88) prepared by this method yielded a single band on an SDS gel after staining with Coomassie blue, and was used for injection without further purification. However, MBP-LAP2 (298–373) required further purification by FPLC chromatography on a Mono S column (Pharmacia Biotechnology, Inc.) to yield a single band of the expected size. For this, partially purified MBP-LAP2 (298–373) eluted from the amylase beads was first dialyzed into a buffer containing 20 mM Tris, pH 8.0, and then was loaded onto the Mono S column, which was eluted with a 0–500 mM NaCl gradient in the same buffer. Essentially pure MBP-LAP2 (298–373) eluted at 200 mM NaCl. His-tagged protein LAP2 (1–398) (provided by Dr. C. Fritze) was expressed in *E. coli* strain BL21 and purified by Ni-NTA agarose beads (QIAGEN Inc., Chatsworth, CA) according to the manufacturer's instructions.

All proteins used for microinjection were concentrated to 1–2 mg/ml by a collodion bag system using a 25-kD cutoff membrane (Schleicher & Schuell, Inc., Keene, NH) and were dialyzed against a solution containing 10 mM Hepes-KOH, pH 7.4, 120 mM KCl for microinjection. The dialyzed proteins were aliquoted and stored at −20°C.

Cell Culture and Microinjection

HeLa and NRK cells were grown at 37°C in a humidified incubator con-

taining 5% CO_2 in DME containing high glucose (GIBCO BRL, Gaithersburg, MD), 10% FBS (Hyclone Laboratories, Logan, UT) and 100 U/ml penicillin and streptomycin (GIBCO BRL). The day before microinjection, the cells were plated in 35-mm tissue culture plates (Corning Glass Works, Corning, NY) containing glass coverslips (Fisher Scientific Co., Pittsburgh, PA). All recombinant LAP2 fragments were microinjected at a concentration of 1 mg/ml in the experiments shown. However, we obtained very similar results by injecting the LAP2 fragments at a concentration of 0.1 mg/ml. Protein samples were centrifuged for 15 min at 13,000 *g* before microinjection. The microinjected cells were identified by coinjecting the LAP2 fragments with either 0.2 mg/ml purified rabbit IgG, or with 0.2 mg/ml rhodamine, or fluorescein-conjugated rabbit IgG (Becton and Dickinson, Co., Mountain View, CA). The rabbit IgG was detected by immunofluorescent staining.

Experiments involving microinjection of LAP2 fragments into mitotic HeLa or NRK cells used exponentially growing cultures. Metaphase cells were selected for injection by their appearance in phase contrast microscopy. For experiments involving injection of synchronized interphase (G1 or S phase) HeLa cells, we first obtained synchronized mitotic populations. For this, HeLa cells growing on 150-cm^2 plates were incubated in medium containing 2 mM thymidine (Aldrich Chemical Co., Milwaukee, WI) for 11 h to accumulate cells in S phase. The thymidine-containing medium was replaced by normal growth medium for an additional 2 h, and the cells were then incubated for another 11 h in medium containing 0.075 μg/ml nocodazole (Sigma Chemical Co., St. Louis, MO) to accumulate cells in M phase. M phase cells were then selected by mechanical shake off, and were replated in normal growth medium in 35-mm plates containing coverslips. In experiments where DNA replication was monitored by bromodeoxyuridine (BrdU) incorporation, cells were plated in medium containing 10 μM BrdU after the shake off; or alternatively, were pulse labeled for 30 min in medium containing 10 μM BrdU before fixation in 4% formaldehyde for immunofluorescence microscopy.

For analysis of nuclear protein import, cells that had been injected with rhodamine-conjugated rabbit IgG were injected with FITC-conjugated NLS-BSA (1 mg/ml), prepared as described (Paschal and Gerace, 1995) and kindly provided by Dr. A. Dickmanns (The Scripps Research Institute). The cells were fixed for examination by fluorescence microscopy after 30 min incubation at 37°C.

Antibodies

Lamins A/C and B were detected with polyclonal guinea pig antibodies (Burke and Gerace, 1986). LAP1, LAP2, and nuclear pore complex glycoproteins were detected with the RL13 (Senior and Gerace, 1988), RL29 (Foisner and Gerace, 1993), and RL1 (Snow et al., 1987) mAbs respectively. The relevant hybridomas were grown in EXCELL 300 serum-free medium (JRH Biosciences, Lenexa, KS); and mAbs were purified from the culture supernatants by ammonium sulfate precipitation followed by chromatography on a protein G column (Pharmacia Biotechnology). The anti-LAP2 antibody (RL29) specifically recognizes LAP2 (thymopoietin β) but not the other two characterized proteins (thymopoietins α and γ) that arise from alternative splicing of the primary LAP2 transcript (Harris et al., 1994).

Immunofluorescence Microscopy

The localization of various NE proteins in injected cells was determined by indirect immunofluorescence microscopy (Yang et al., 1997). HeLa cells growing on glass coverslips were fixed in 4% formaldehyde in PBS for 6 min at room temperature, permeabilized with 0.2% Triton X-100 in PBS for another 6 min, and then treated with PBS containing 0.2% gelatin. Cells were then incubated with primary antibodies diluted in PBS/gelatin for 1 h at room temperature, washed in PBS, incubated for another 40 min at room temperature with secondary antibodies diluted in PBS/gelatin, and then finally washed in PBS. mAbs RL13 (mouse IgG), RL29 (hamster IgG), and RL1 (mouse IgM) were used at 25 μg/ml, and were detected with fluorescein-conjugated, sheep anti–mouse IgG or IgM, or fluorescein-conjugated, donkey anti–hamster IgG (Molecular Probes Inc., Eugene, OR). Polyclonal guinea pig antibodies were detected with fluorescein-conjugated, donkey anti–guinea pig IgG (Becton and Dickinson, Co.). BrdU incorporation was determined by immunofluorescent staining using FITC-conjugated anti-BrdU antibodies (Becton and Dickinson, Co.) according to the manufacturer's instructions. The specimens were mounted in slowfade antifade solution (Molecular Probes Inc.) and examined with an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) or a laser-scanning confocal microscope (MRC-600; Bio-Rad Laboratories, Hercules, CA).

EM

To carry out EM on injected cells, cells were plated in 35-mm plates containing pieces of Aclar embedding film (Ted Pella, Inc., Redding, CA). Cells were coinjected with MBP-LAP2 (298–373), 5-nm colloidal gold particles (for identification of injected cells in EM sections), and FITC-conjugated, 150-kD dextran (for localization of injected cells on the film coverslips). After incubation at 37°C for appropriate times, the cells attached to the embedding film were fixed for 30 min in 2% glutaraldehyde, 1% tannic acid in PBS, and then were processed for silver enhancement as described (Barry, 1995) using a silver enhancement kit (Ted Pella, Inc.). The microinjected cells were identified under the fluorescence microscope by FITC fluorescence and marked by inscribing a frame around them or removing surrounding uninjected cells with a microinjection needle. The cells were then fixed with 1% OsO_4 for 1 h at room temperature and relevant portions of the Aclar film were excised. Samples were dehydrated and embedded in Epon 812 resin as described (Guan et al., 1995), with the film and attached cells resting in the bottom of a plastic microtiter well. After polymerization of the resin, the Epon block was removed from the plastic microtiter well. The relevant marked areas in the Aclar film (containing microinjected cells) were identified with a dissecting microscope, and thin sections containing these areas were obtained. Thin sections were stained with 2% uranyl acetate for 1 min. Micrographs were recorded with a Hitachi 600 electron microscope (Hitachi Instruments Inc., San Jose, CA) at 80 kV. The injected cells were identified by the coinjected gold particles, which appeared as black spots in the cells because of the silver enhancement. For determining the relative density of nuclear pore complex (NPCs) in the NE of cells injected with LAP2 (1–88) or LAP2 (298–373), we selected micrographs containing cross-sections of the NE, and scored the number of NPCs per contour length of the NE (145 and 459 NPCs were scored for cells injected with LAP2 [1–88] and LAP2 [298–373], respectively).

Nuclear Volume Calculation

To calculate the nuclear volume in synchronized cells, cultures were fixed at appropriate times with formaldehyde and stained for immunofluorescence microscopy with anti–lamin B polyclonal antibodies to delineate the NE. A Z series of fluorescent images was collected with the confocal microscope through the entirety of the nucleus from top to bottom. In most cases, 20 sections were collected in the Z series, and each section was 0.36-μm thick. In each section, the pixel number in the nucleus (whose boundary was marked by lamin B) was calculated using Photoshop (Adobe Systems, Mountain View, CA), and then was converted to the nuclear area on the basis of the magnification. The fractional nuclear volume in each section was calculated as the intranuclear area multiplied by the section thickness. The total volume of each nucleus was obtained by summing the fractional nuclear volumes in all sections containing a portion of the nucleus. The average nuclear volume of 20 cells was determined for each experiment. When this method was used to calculate the volume of fluorescent beads of a known size, the calculated volume closely matched the actual value.

Results

Effect of Nucleoplasmic Segments of LAP2 on Nuclear Assembly and Growth at the End of Mitosis

To study a possible role of LAP2 in NE reassembly at the end of mitosis, we injected metaphase HeLa cells with a variety of monoclonal and polyclonal antibodies raised against this protein. None of the antibodies prevented assembly of LAP2 in the NE, nor did they have any detectable effects on NE reformation (data not shown). As an alternative strategy for disrupting LAP2 function, we investigated whether various fragments from the nucleoplasmic domain of rat LAP2 would have a dominant negative effect on NE reformation or nuclear structure when injected into metaphase HeLa cells. We expected that these

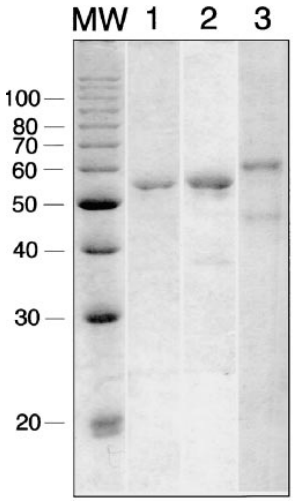

Figure 1. Analysis of LAP2 fragments used for microinjection by SDS-PAGE. Samples of recombinant proteins used for microinjection were electrophoresed on a 10% SDS gel, that was stained with Coomassie blue. *MW*, molecular weight standards; lane *1*, MBP-LAP2 (298–373); lane *2*, MPB-LAP2 (1–88); lane *3*, His-tagged LAP2 (1–398).

soluble LAP2 fragments might act as competitive inhibitors of the endogenous, membrane-integrated LAP2.

In initial experiments, we injected the mitotic cells with a recombinant protein comprising nearly the entire nucleoplasmic domain of LAP2 (residues 1–398; Furukawa et al., 1995) (Fig. 1, lane *3*) or with various control proteins, and examined the cells 2 h later. Under these conditions, the cells that had been injected with control proteins (IgG or GST), completed mitosis, and by phase contrast microscopy appeared as pairs of daughter cells with well-defined G1 nuclei containing decondensed chromatin (Fig. 2, *B* and *F*; and data not shown). Interestingly, the cells that had been injected with LAP2 (1–398) (Fig. 2, *A* and *E*) completed mitosis like the control cells, but the nuclei in these cells had a markedly reduced size, even though they appeared to contain decondensed chromatin and nucleoluslike phase dense bodies. Immunofluorescent staining showed that despite the reduction in nuclear size, lamins A/C and B assembled in the nuclei of the LAP2 injected cells, similar to the control cells (Fig. 2, compare *A* and *E* to *B* and *F*). Lamin B appeared in a distinct nuclear rim, whereas lamins A/C appeared both in a nuclear rim and in intranuclear foci. These results on lamins A/C localization are in accord with previous findings showing that a fraction of lamin A appears in intranuclear foci as well as the NE in some cultured mammalian cells in early G1 phase, although all detectable lamin A becomes localized at the NE later in G1 phase (Bridger et al., 1993; Gerace, L., unpublished results).

We also carried out immunofluorescent staining of injected cells to localize LAP1, LAP2, and a group of NPC glycoproteins recognized by the RL1 mAb. We found that these proteins were all assembled in the NE of cells injected with LAP2 (1–398) in a manner indistinguishable from the control cells (data not shown; see below). Thus, a protein fragment comprising the nucleoplasmic domain of LAP2 appears to inhibit nuclear volume increase after nuclear reformation, but does not have a detectable effect on assembly of a variety of NPC and lamina markers in the NE (including LAP2).

The inability of the nucleoplasmic domain of LAP2 to inhibit the targeting of endogenous LAP2 to the NE at the

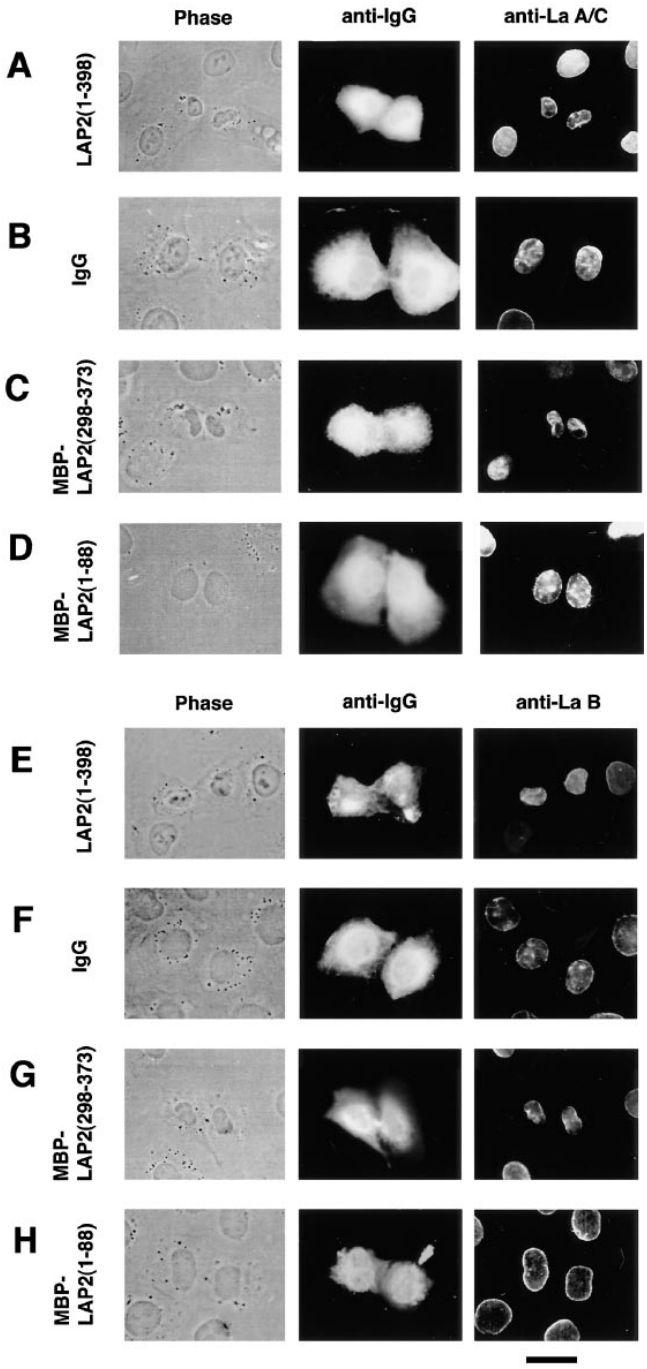

Figure 2. Effect of microinjected LAP2 fragments on nuclear reformation and lamin assembly at the end of mitosis. Metaphase HeLa cells were injected with a variety of proteins and incubated for 2 h at 37°C before fixation and immunofluorescent staining. All injectates contained rabbit IgG to mark the injected cells. In addition, the injectates contained: *A* and *E*, LAP2 (1–398); *B* and *F*, rabbit IgG only; *C* and *G*, MBP-LAP2 (298–373); *D* and *H*, MBP-LAP2 (1–88). Shown are phase contrast images (*left column*), or epifluorescence images staining with anti–rabbit IgG (*middle column*), and with antilamins (*right column*; *A–D*, antilamins A/C; *E–H*, antilamin B). Bar, 20 μm.

end of mitosis has a number of possible explanations. For example, it is possible that the LAP2 fragment, which lacks the transmembrane and lumenal domains of intact LAP2, does not interact with all the NE-binding sites of the endogenous LAP2, or that the affinity of the LAP2 fragment for the NE-binding sites is significantly lower than the affinity of the intact LAP2 (e.g., because of the quaternary structure of the latter). However, this is not because of the injection of a heterologous (rat) LAP2 fragment into human (HeLa) cells, since the same phenotype (inhibition of nuclear growth but not the assembly of LAP2 into the NE) was obtained by injecting rat LAP2 (1–398) into NRK (rat) cells (data not shown). The lack of an effect of LAP2 (1–398) (or the minimal lamin binding region of LAP2, see below) on reassembly of lamins in our system does not rule out the possibility that these proteins could affect lamin assembly with other experimental conditions, since the intranuclear concentration of the LAP2 fragments might be limiting during the short period after nuclear membrane reformation when the assembly of most nuclear lamins takes place in cultured mammalian cells (Yang et al., 1997).

We have recently delineated the lamin- and chromatin-binding regions in LAP2 (Furukawa K., C. Fritze, and L. Gerace, manuscript submitted for publication). Using a yeast two-hybrid approach, we have found that a minimal lamin B–binding region occurs between residues 298–373 of LAP2. Moreover, using an immunofluorescent chromosome binding assay with recombinant segments of LAP2, we determined that the chromatin-binding region of LAP2 lies between residues 1–88. We took advantage of this information to investigate whether the ability of the nucleoplasmic domain of LAP2 to inhibit the increase in nuclear volume could be assigned to a region that we have implicated in either lamin or chromatin binding.

For this purpose we prepared recombinant proteins containing the MBP fused to either the chromatin-binding region or the lamin B–binding region of LAP2 (Fig. 1, lanes *1* and *2*). Microinjection analysis similar to that described above showed that cells injected with MBP-LAP2 (1–88) underwent normal mitosis and assembly of lamins, and were indistinguishable in their nuclear size from the control cells injected only with IgG (Figs. 2, *D* and *H*, and 3, *A–C*). By contrast, cells injected with MBP-LAP2 (298–373) showed strong inhibition of nuclear growth similar to that seen with LAP2 (1–398), even though assembly of lamins was normal (Figs. 2, *C* and *G*, and 3, *D–F*). In initial studies we obtained very similar results with GST fusion proteins containing each of the three LAP2 fragments (data not shown). However, since GST is a dimer and the dimeric GST fusion proteins could potentially cross-link lamins, we believed it important to perform the experiments with MBP fusions, which are monomers.

We then used confocal fluorescence microscopy to compare the assembly of LAP1, LAP2, and the RL1 NPC antigens in the cells injected with MBP-LAP2 (298–373) and MPB-LAP2 (1–88) (Fig. 3). LAP1 and LAP2, as well as the RL1 antigens, were localized at the nuclear periphery of cells injected with MBP-LAP2 (298–373), similar to cells injected with MBP-LAP2 (1–88). Thus, although nuclear volume increase is inhibited when mitotic HeLa cells are injected with MBP-LAP2 (298–373), a variety of nuclear lamina and NPC proteins still become assembled in the NE.

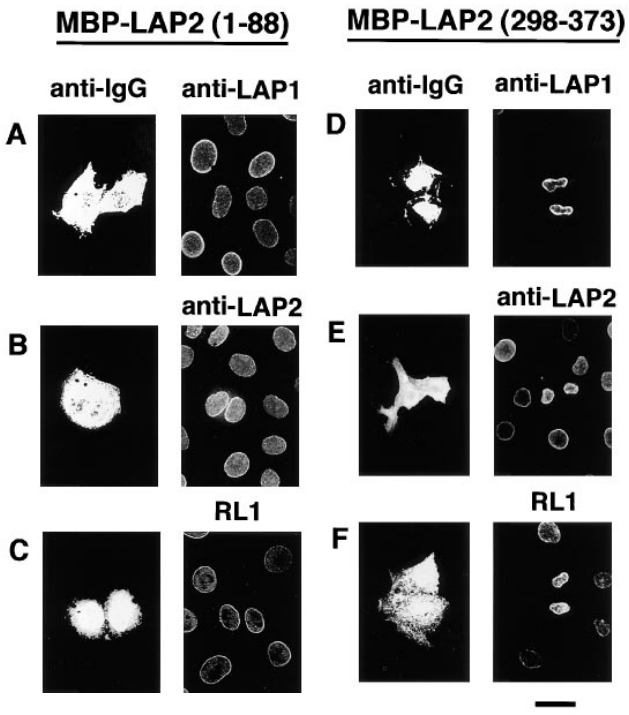

Figure 3. Effect of microinjected LAP2 fragments on reassembly of LAPs and pore complex proteins at the end of mitosis. Metaphase HeLa cells were injected with rabbit IgG plus MBP-LAP2 (1–88) (*A–C*) or MPB-LAP2 (298–373) (*D–F*). After incubation for 2 h at 37°C, cells were fixed and stained for immunofluorescence microscopy. Shown are confocal fluorescence images of staining with anti–rabbit IgG (*left image* in *A–F*) and with antibodies against various NE proteins (*right image* in *A–F*): *A* and *D*, anti-LAP1; *B* and *E*, anti-LAP2; *C* and *F*, RL1. Bar, 20 μm.

In further studies, we used thin section EM to examine the ultrastructure of nuclei in the cells that had been injected with LAP2 (298–373) or LAP2 (1–88) as above. The nuclei of cells injected with LAP2 (298–373) usually had a convoluted surface and a shape similar to that of late telophase nuclei (Fig. 4 *B*), consistent with their appearance in confocal light microscopy (Fig. 3). This contrasted with the more spherical shape of nuclei in cells injected with LAP2 (1–88) (Figs. 3 and 5 *B*). In cells injected with LAP2 (298–373), the chromatin was extensively decondensed, and lined the inner surface of the NE in an almost continuous fashion, except for interruptions at the pore complexes (Fig. 4 *C*). This was similar to cells injected with LAP2 (1–88) (Fig. 5 *C*). Finally, the NE of cells injected with LAP2 (298–373) had a normal double membrane and characteristic NPCs, like the cells injected with LAP2 (1–88). One difference between the two types of injected cells, however, was the density of NPCs in the NE, which was 1.7-fold greater in cells injected with LAP2 (298–373), compared to cells injected with LAP2 (1–88), as determined by quantitative analysis (see Materials and Methods). The difference in density of pore complexes is apparent in tangential sections of the nuclear surface of these two classes of injected cells (compare Figs. 4 *A* and 5 *A*). This difference is probably because of continued assembly of NPCs in NEs

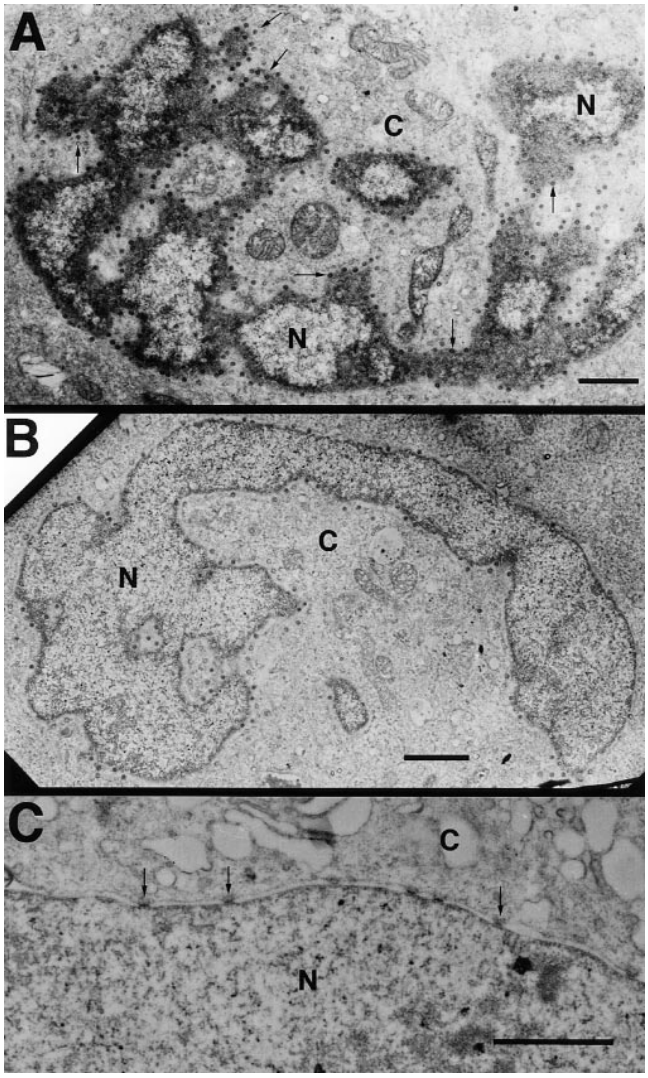

Figure 4. Electron microscopy of G1 phase cells injected with LAP2 (298–373) at metaphase. Shown are thin section electron micrographs depicting the nucleus of cells that were injected with LAP2 (298–373) at metaphase and returned to culture for 2 h. Nuclear (*N*) and cytoplasmic (*C*) areas are indicated. *A* shows a tangential section of the nuclear surface, whereas *B* and *C* show cross-sections of the nucleus. Nuclear pore complexes are indicated by arrows. Bars, 1 μm.

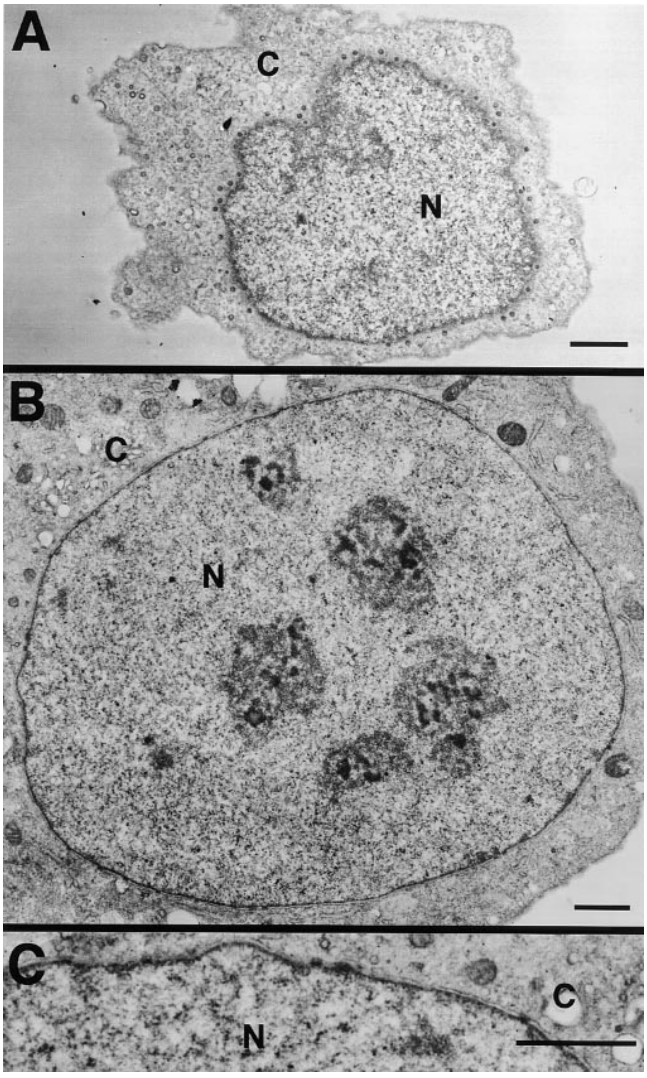

Figure 5. EM of G1 phase cells injected with LAP2 (1–88) at metaphase. Shown are thin section electron micrographs depicting the nucleus of cells injected with LAP2 (1–88) at metaphase and returned to culture for 2 h. Nuclear (*N*) and cytoplasmic (*C*) areas are indicated. *A* shows a tangential section of the nuclear surface, whereas *B* and *C* show cross-sections of the nucleus. Bars, 1 μm.

in which the increase in surface area was inhibited by injection of LAP2 (298–373).

We also examined the nuclear transport capacity of cells that had been injected with the LAP2 fragments. For this we coinjected a fluorescent reporter protein containing basic amino acid–type nuclear localization signals (FITC-labeled NLS-BSA; see Materials and Methods) into the metaphase cells and examined the localization of the fluorescent import substrate after 2 h. We found that after 30 min the transport substrate was highly concentrated in the nuclei of cells injected with LAP2 (298–373), like cells injected with LAP2 (1–88) (data not shown). Thus, the nuclei of the injected cells appear to be intact and are capable of actively importing nuclear proteins. Similar results were obtained when interphase cells were injected with these LAP2 fragments (see Fig. 8; below). In conclusion, cells injected with the lamin-binding fragment of LAP2 (298–373) assemble a NE with apparently normal structural and functional features. Aside from an increase in NPC density in the NE, the only clear difference in these injected cells was a marked inhibition of nuclear growth.

Inhibition of Interphase Nuclear Volume Increase by the Lamin-binding Fragment of LAP2

We next examined whether the lamin-binding domain of LAP2 could inhibit the substantial increase in nuclear volume that occurs during interphase in cycling cells (Maul et al., 1972; Fidorra et al., 1981). For this experiment, the LAP2 fragment was injected into early G1 phase cells, which have a fully assembled and functional NE. Mitotic HeLa cells were selected by mechanical shake off from nocodazole-

arrested cultures and were returned to culture in the absence of nocodazole for 2 h to allow progression into G1. The cells then were injected with MBP-LAP2 (298–373) or the control fragment, MBP-LAP2 (1–88), and nuclear volume was examined 10 h later. Immunofluorescent staining demonstrated that both proteins entered the nucleus quite rapidly; at 2 h after microinjection, MBP-LAP2 (1–88) was present in both the nucleus and the cytoplasm at similar levels, whereas MBP-LAP2 (298–373) was somewhat more concentrated in the nucleus than in the cytoplasm at this time (data not shown). The mechanism by which the fusion proteins enter the nucleus (i.e., passive diffusion or active transport) is unknown.

Phase contrast microscopy demonstrated that at 10 h after microinjection, the nuclei of cells injected with MBP-LAP2 (298–373) were very small compared to the nuclei of uninjected cells (e.g., Fig. 6 *A*), whereas the nuclei of cells injected with MBP-LAP2 (1–88) were roughly the same size as the nuclei of control cells (e.g., Fig. 6 *C*). We quantitatively analyzed the changes in nuclear volume that took place in cells microinjected with MBP-LAP2 (298–373) or in injected controls during this 10-h period (Table I). We determined that the nuclei of uninjected HeLa cells on average underwent an ~threefold increase in nuclear volume between 2 and 12 h after mitosis. In sharp contrast, the nuclei of cells injected with MBP-LAP2 (298–373) at 2 h after mitosis had virtually the same volume 10 h later. However, if cells were injected with MBP-LAP2 (298–373) at 10 h after mitosis, 2 h later they had the same volume as uninjected cells at 12 h after mitosis. Therefore, MBP-LAP2 (298–373) acts to prevent an increase in nuclear volume during interphase, but cannot induce a decrease in the volume of nuclei that have already grown by progression through the cell cycle.

Inhibition of Progression into S Phase by the Lamin-binding Fragment of LAP2

We next investigated whether the lamin-binding fragment of LAP2 had an effect on progression of cells into S phase, in addition to its inhibition of nuclear volume increase during interphase. Under our synchronization conditions, the percentage of cells in S phase was greatest at 10–12 h after release from the nocodazole block (Fig. 7 *A*). Early G1 phase HeLa cells (2 h after nocodazole release) were injected with MBP-LAP2 (298–373) or MBP-LAP2 (1–88), and were incubated for another 10 h. Cells were then pulse labeled with BrdU (a thymidine analogue) and examined by immunofluorescent staining to detect the incorporation of BrdU into nuclear DNA (Figs. 6, *A* and *C*). A majority of cells injected with MBP-LAP2 (1–88) incorporated BrdU in their nucleus (Fig. 6 *C*), whereas almost no nuclei of cells injected with LAP2 (298–373) showed BrdU incorporation (Fig. 6 *A*). Quantitative analysis demonstrated that only 8% of the cells injected with LAP2 (298–373) incorporated BrdU, in contrast to 53% of the cells injected with LAP2 (1–88) and 55% of the uninjected cells (Fig. 7 *B*). A similar result was obtained when MBP-LAP2 (298–373)–injected cells were cultured continuously in BrdU-containing medium for the 10 h after microinjection, instead of being pulse labeled with BrdU at the end of this period (data not shown).

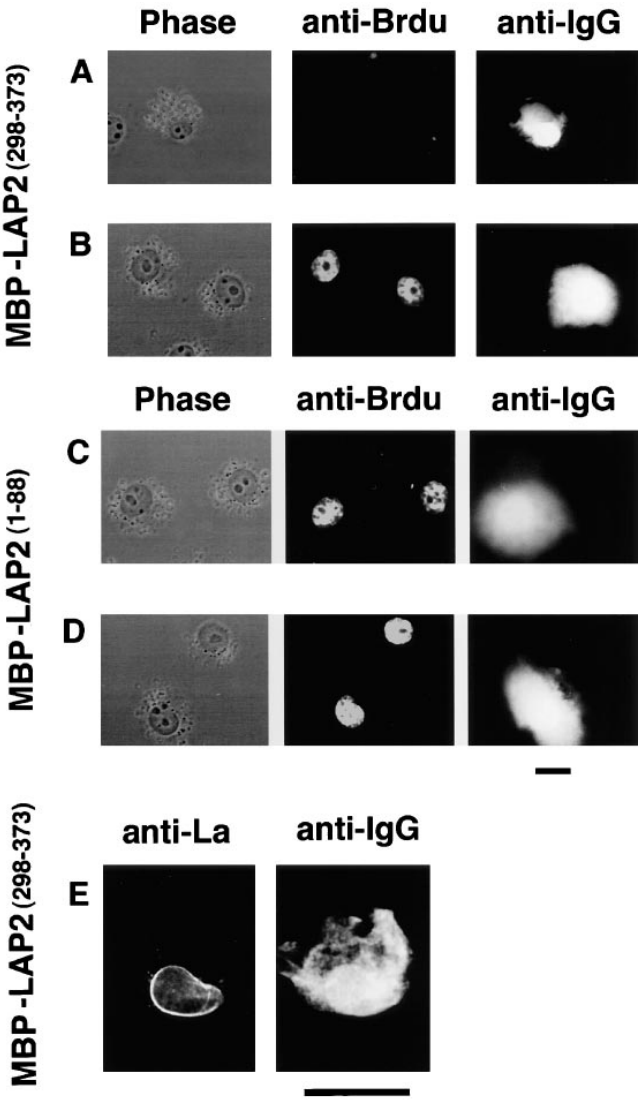

Figure 6. Effect of injected LAP2 fragments cells on interphase nuclear growth and progression to S phase. Mitotic HeLa cells were obtained by shake off, and returned to culture. Some cells were injected with MBP-LAP2 (298–373) either 2 h (*A* and *E*) or 10 h (*B*) later. Other cells were injected with MBP-LAP2 (1–88) either 2 h (*C*) or 10 h (*D*) later. All injectates also contained rabbit IgG. 12 h after the mitotic cells were returned to culture, the cultures in *A–D* were pulse labeled with BrdU for 30 min, and were fixed and processed for immunofluorescence microscopy as indicated. The culture in *E* was fixed at 12 h. Shown are phase contrast images (*A–D*, *left column*), or epifluorescence images of staining with anti-BrdU (*A–D*, *middle column*), and with anti–rabbit IgG (*A–D*, *right column*). *E* shows confocal fluorescence images of staining with antilamins A/C (*left*) and with anti-IgG (*right*). Bars, 10 μm.

To examine whether the MBP-LAP2 (298–373) had an inhibitory effect on ongoing DNA replication, cultures were incubated for 10 h after the nocodazole release, to the time when a maximum percentage of cells were in S phase, and then were injected with MBP-LAP2 (298–373) or MBP-LAP2 (1–88). 2 h later, the cells were pulse labeled with BrdU and examined by immunofluorescent staining (Figs. 6, *B* and *D*). Under these conditions, a ma-

Table I. Effect of LAP2 (298–373) on Nuclear Volume Increase during the Cell Cycle

Treatment	None	None	Injection with LAP2 (298–373) at 2 h	Injection with LAP2 (298–373) at 10 h
Sampling time*	2 h	12 h	12 h	12 h
Nuclear volume‡ (μm^3)	312 ± 183	895 ± 254	350 ± 178	882 ± 215

*Time after release from nocodazole block.
‡Average of 20 cells, determined as in Materials and Methods.

jority of the cells injected with LAP2 (298–373) incorporated BrdU (Fig. 6 *B*), similar to cells injected with LAP2 (1–88) (Fig. 6 *D*). Quantitative analysis showed that 53% of the cells injected with LAP2 (298–373) were labeled with BrdU, which does not differ significantly from the uninjected control (Fig. 7 *B*). This demonstrates that the lamin-binding fragment of LAP2 does not inhibit ongoing DNA replication once cells have reached S phase. Rather, inhibition of DNA replication occurs only when the fragment is injected into early G1 phase cells and a clamp is imposed on nuclear volume.

We performed a series of additional experiments to investigate the basis for the inhibition of DNA replication in these injected cells. First, we carried out immunofluorescent staining to examine the localization of nuclear lamins at 12 h after mitosis in the cells that had been injected with LAP2 (298–373) 2 h after the nocodazole release. This experiment showed that lamins A/C remain highly concentrated at the NE in these cells (Fig. 6 *E*), similar to uninjected cells. Thus LAP2 (298–373) does not mediate inhibition of DNA replication by inducing lamin disassembly. Furthermore, the lack of cytoplasmic or intranuclear aggregates of lamins suggest that newly synthesized lamins are normally assembled in the NE. Next, we investigated whether there was an obvious deficiency in nuclear protein import in the injected cells. In one experiment, exponentially growing cells were injected with LAP2 (298–373) or LAP2 (1–88), and 2 h later were injected with a nuclear import substrate (FITC-NLS-BSA) (Figs. 8, *A* and *B*). In a second experiment, early G1 phase cells were injected with LAP2 (298–373) or LAP2 (1–88), and 10 h later were injected with FITC-NLS-BSA (Figs. 8, *C* and *D*). In both of these experiments, after 30 min the substrate became highly concentrated in the nucleus of the cells injected with LAP2 (298–373) (Figs. 8, *A* and *C*), similar to the cells injected with LAP2 (1–88) (Figs. 8, *B* and *D*). All of the injected cells showed rapid import kinetics, since most of the transport substrate had accumulated in the nucleus within 5–10 min of injection (data not shown). This demonstrates that injection of LAP2 (298–373) into interphase cells did not detectably disrupt either the structure of the NE or its capacity for nuclear import.

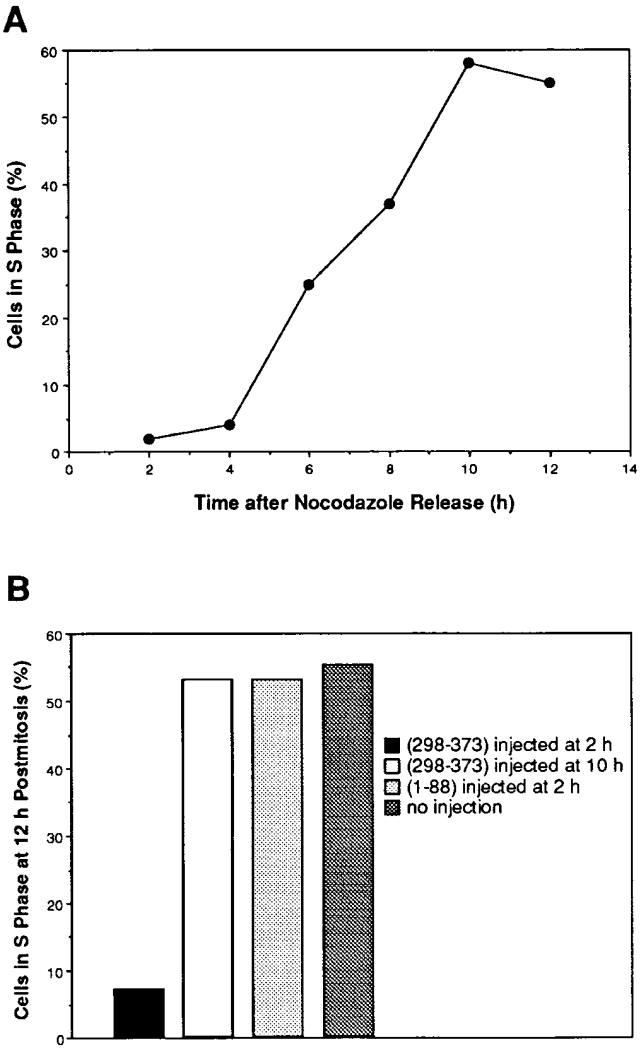

Figure 7. Quantitative analysis of effect of injected LAP2 (298–373) on progression of cells into S phase. (*A*) Kinetics of S phase progression. After selection of mitotic HeLa cells by shake off, the cells were returned to culture in medium containing BrdU and were fixed at various times and processed for immunofluorescence microscopy with anti-BrdU. The percentage of cells showing BrdU staining is indicated. (*B*) Mitotic cells obtained by shake off were returned to culture, and cells were injected with LAP2 (298-373) at 2 h (*black bar*), or at 10 h (*white bar*), or with LAP2 (1–88) at 2 h (*lightly shaded bar*). The cultures injected with LAP2 (298–373) and LAP2 (1–88) at 2 h, and a control uninjected culture (*darkly shaded bar*), were grown in medium containing BrdU from 2–12 h, and the cells were then fixed and processed for immunofluorescence microscopy with anti-BrdU. The culture injected with LAP2 (298–373) at 10 h was pulse labeled with BrdU for 30 min at 12 hr, and the cells were then fixed and stained with anti-BrdU. Shown are the percent of each population that incorporated BrdU.

Discussion

Functions of LAP2 Related to Nuclear Lamina Dynamics

We have found that microinjection of a soluble recombinant protein comprising the nucleoplasmic domain of LAP2 into metaphase HeLa cells strongly inhibits the increase in nuclear volume that occurs in early G1 phase, even though the cells assemble an apparently normal, transport-competent NE, containing LAP1, LAP2, lamins, and NPCs. This phenotype appears to be due to lamin binding by the LAP2 fragment, because an identical effect is achieved with the 76–amino acid segment of the nucleo-

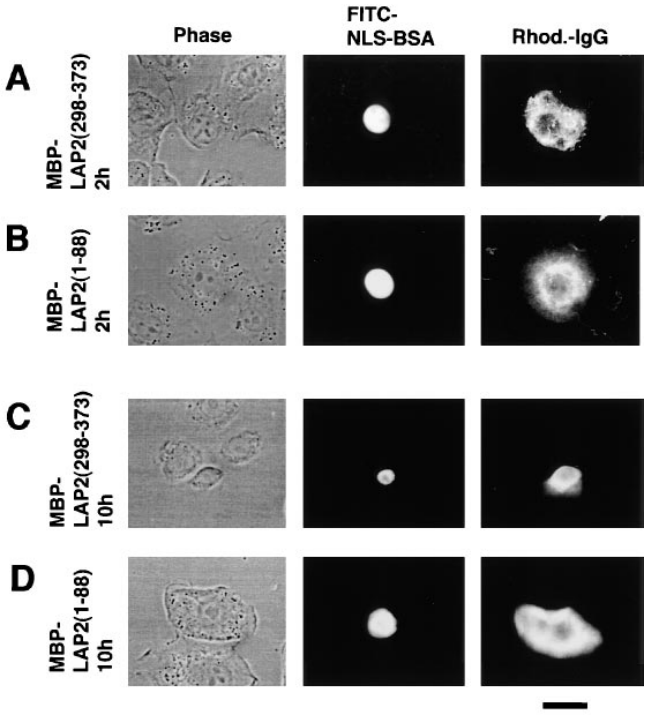

Figure 8. Analysis of nuclear protein import in interphase cells injected with LAP2 fragments. (A and B) Interphase HeLa cells in exponentially growing populations were injected with either MPB-LAP2 (298–373) (*A*) or MBP-LAP2 (1–88) (*B*) together with fluorescent rabbit IgG. After 2 h, the injected cells were given a second injection with FITC-NLS-BSA. The cells were fixed 30 min later and examined. (*C* and *D*) Mitotic HeLa cells were selected by mitotic shake off and were returned to culture. After 2 h, they were injected with either MPB-LAP2 (298–373) (*C*) or MBP-LAP2 (1–88) (*D*). 10 h later, the injected cells were given a second injection with FITC-NLS-BSA. The cells were fixed 30 min later and examined. Shown are phase contrast images (*left column*), epifluorescence images of FITC-NLS-BSA (*middle column*), and epifluorescence images of rhodamine-IgG (*right column*). Bars, 20 μm.

plasmic domain of LAP2 (LAP2 [298–373]) that represents its minimal lamin-binding region. By contrast, no effect is seen with the LAP2 fragment that contains its chromatin-binding domain (LAP2 [1–88]).

The injection of LAP2 (298–373) into early G1 phase cells produces an effect similar to that seen in mitotic cells. The LAP2 fragment completely inhibits the ~threefold increase in nuclear volume that normally occurs during the ensuing 10 h of the cell cycle, without detectably perturbing NE structure or nuclear import. In this case, inhibition of nuclear growth by the LAP2 fragment is clearly unrelated to mitotic NE reassembly, because the G1 cells have a functional interphase NE at the time of injection.

We believe that the clamp on nuclear volume imposed by the lamin-binding fragment of LAP2 most likely is because of its interference with the normal structural dynamics of the nuclear lamina, which in turn could inhibit an increase in NE surface area and nuclear volume (see below). In principle, growth of the lamina may be driven by reorganization of assembled lamins and/or by insertion of new lamin subunits (Gerace et al., 1984; Gerace and Burke, 1988). The injected LAP2 fragments do not appear to interfere with incorporation of lamins in the NE at the end of mitosis or during interphase, but could nevertheless modify the pathway of lamin insertion, and indirectly influence the expansion of the assembled lamina. Alternatively, they could directly block expansion of the nuclear lamina by binding to a site on nuclear lamins required for lamina growth. However, we can clearly exclude the possibility that the microinjected LAP2 fragments are freezing the structure of the lamina by cross-linking lamins, since the MBP fusions we used for these studies are monomers.

The microinjected LAP2 fragments probably do not bind to the lamina in a fashion identical to the endogenous LAP2. This is because the endogenous LAP2 is a membrane-integrated protein, and unlike the soluble LAP2 fragments, should have topological access only to the surface of the lamina filaments facing the inner nuclear membrane. Nevertheless, we consider it likely that the injected LAP2 fragments are mimicking a normal function of LAP2. Thus, our data suggest that one function of LAP2 is to stabilize nuclear lamina structure and/or negatively regulate lamina growth during interphase. For interphase lamina expansion to occur, it may be necessary to transiently weaken or abolish the lamin–LAP2 binding in localized regions of the lamina. This could be achieved by a reversible, postsynthetic modification(s) of LAP2. Such an effect could be achieved by phosphorylation, since phosphorylation of LAP2 is known to inhibit the lamin–LAP2 interaction during mitosis (Foisner and Gerace, 1993).

Role of the Lamina in NE Growth during Interphase

A number of previous investigations have provided evidence to support the notion that nuclear volume increase involves the nuclear lamina. Injection of polyclonal antilamin antibodies into mitotic cultured cells was found to prevent most lamin assembly and to arrest nuclei in an early telophase configuration, in which the chromosomes were surrounded by a double membrane but remained in a highly condensed state (Benavente and Krohne, 1986). Further support for an involvement of the lamina in nuclear growth came from studies involving in vitro nuclear assembly with *Xenopus* egg extracts. It was found that immunological depletion of the major egg lamin L_{III} (Newport et al., 1990; Goldberg et al., 1995), or inhibition of L_{III} assembly by the addition of anti-L_{III} antibodies (Meier et al., 1991) or a dominant-negative lamin mutant (Spann et al., 1997) resulted in the assembly of import-competent nuclei with intact NEs, but significantly impaired nuclear growth. However, it was unclear whether a residual nuclear lamina involving the minor egg lamin isotypes (Lourim and Krohne, 1993; Lourim et al., 1996) was assembled in the NE under these conditions. Our studies complement and extend these earlier studies on the inhibition of lamin assembly. In our experiments involving injection of the lamin-binding fragment of LAP2 into G1 cells, the fragment functioned in the context of a structurally normal NE, and most likely acted through inhibition of lamin dynamics rather than on assembly. Thus, the results in the present study strongly support the notion that the increase in nuclear volume that occurs in growing cells during interphase involves the nuclear lamina, at least in part. The growth of the nuclear

lamina could be regulated both by intrinsic mechanisms (involving insertion and rearrangement of lamins, and modulation by lamina-associated polypeptides like LAP2 [discussed above]), and by extrinsic mechanisms (such as changes in the volume or higher order conformation of chromatin) (i.e., Nicolini et al., 1984).

A Nuclear Volume Checkpoint for S Phase Progression?

In previous studies with *Xenopus* egg extracts where assembly of lamin L_{III} in the NE was perturbed by various means, an inhibition of DNA replication was observed (Newport et al., 1990; Meier et al., 1991; Goldberg et al., 1995; Spann et al., 1997) in addition to a deficiency in nuclear growth (see above). However, in some of these studies, essential DNA replication factors became concentrated in intranuclear aggregates containing lamins (Meier et al., 1991; Spann et al., 1997). This suggests that inhibition of DNA replication in these cases involved removal of replication proteins from their normal site of function, at least in part. Nevertheless, entry into S phase may be dependent on assembly of the nuclear lamina and certain lamina functions, such as chromatin anchoring to the NE (Hutchison et al., 1994; Zhang et al., 1996).

Our studies showed that injection of the lamin-binding fragment of LAP2 into HeLa cells in early G1 phase strongly inhibited progression of cells into S phase, in addition to preventing a nuclear volume increase. However, in contrast to the work with *Xenopus* egg extracts discussed above, the nuclear lamina was not disrupted in our studies involving the microinjected LAP2 fragment. Since LAP2 (298–373) does not bind to chromatin in vitro, or detectably perturb ongoing DNA synthesis, NE integrity, or nuclear import when injected into cells, these experiments suggest that the microinjected LAP2 fragment inhibits progression of cells into S phase by imposing a clamp on nuclear volume rather than by interfering with gross features of nuclear or lamina organization. Nevertheless, we cannot rule out that LAP2 (298–373) has some additional activity other than inhibition of nuclear/lamina growth that is responsible for the inhibition of S phase progression, or that it indirectly affects entry into S phase by displacing some presently uncharacterized proteins from the lamina.

A plausible model suggested by our results is that progression into S phase is dependent on the acquisition of a certain nuclear volume. Consistent with this possibility, a significant increase in nuclear volume occurs in a variety of mammalian cells during G1 phase before entry into S phase (Maul et al., 1972; Yen and Pardee, 1979; Fidorra et al., 1981; this study). This volume requirement for S phase progression could be mediated by two different (and not mutually exclusive) regulatory mechanisms. In one scenario, certain structural features of the nucleus that are directly linked to volume (such as the surface area of the NE/lamina) could be necessary for the generation of an appropriate chromatin substrate for DNA replication. Alternatively, in keeping with the predominant mechanisms that have been described for regulation of cell cycle transitions (Elledge, 1996), our results raise the interesting possibility that eukaryotic cells have a cell cycle checkpoint for entry into S phase that is related to nuclear volume. This would be conceptually similar to the G1 commitment point in yeast called Start that is linked to cell size (Johnston et al., 1977, 1979; Nash et al., 1988). In the future, it will be feasible to use cultured cell lines where the lamin-binding fragment of LAP2 can be inducibly expressed to investigate in detail the possible existence of a nuclear volume checkpoint.

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