The Major Nuclear Envelope Targeting Domain of LAP2 Coincides with Its Lamin Binding Region but Is Distinct from Its Chromatin Interaction Domain*

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LAP2 is an integral protein of the inner nuclear membrane which binds lamins and chromosomes and is suggested to have an important role in nuclear envelope organization. In a previous study we identified an internal 76-amino acid region of LAP2 which is required for stable targeting of the protein to the nuclear envelope. Here, we have mapped the lamin binding region of LAP2 and demonstrate that it coincides with this nuclear envelope targeting domain. In contrast, we found that the portion of LAP2 involved in binding to chromosomes resides in a separate region of the protein near its NH₂ terminus. The minimal lamin binding region of LAP2 is capable of conferring stable nuclear envelope localization when attached to the transmembrane and partial luminal domains of a protein that shows no nuclear envelope targeting activity. This directly supports the notion that a major mechanism for localization of integral membrane proteins at the inner nuclear membrane involves binding to lamins, which would constrain diffusion through the continuous nuclear envelope/endoplasmic reticulum membrane system.

The nuclear envelope (NE)¹ is a specialized region of the ER that forms the nuclear boundary in eukaryocytes (for review, see Refs. 1–4). It consists of a double membrane that is perforated by pore complexes and which is lined by the nuclear lamina in higher eukaryotic cells. The outer nuclear membrane is continuous with the more peripheral ER and is linked to the inner nuclear membrane via a “pore membrane” adjacent to the pore complexes. Whereas the outer membrane is biochemically and functionally similar to peripheral ER, the inner membrane differs markedly from the latter because of its association with the nuclear lamina and its content of specific integral membrane proteins that are mostly absent from the peripheral ER (5, 6).

The nuclear lamina is thought to provide a structural framework for the NE and a chromatin anchoring site at the nuclear periphery (for review, see Refs. 1, 3). The lamina contains a polymer of intermediate-type filament proteins termed lamins as well as a number of more minor lamina-associated polypeptides. Four major lamin subtypes have been identified in mammalian somatic cells, lamins A, B1, B2, and C (for review, see Ref. 3). In higher eukaryocytes, four lamina-associated proteins of the inner nuclear membrane have been identified: lamina-associated polypeptide (LAP)¹ (7, 8), LAP (9, 10), p58/lamin B receptor (LBR) (11, 12), and otefin (13). LAP (8), LAP (10) and LBR (11) are type II integral membrane proteins (14). LAP1 and LAP2 each contains a single predicted transmembrane domain and a large nucleoplasmic region (8, 10). In contrast, LBR, which is homologous to yeast sterol C14 reductase (discussed in Ref. 1), contains eight predicted membrane-spanning regions. Otefin appears to be more peripherally associated with the inner nuclear membrane based on chemical extraction and contains a short hydrophobic segment at its COOH terminus that is not predicted to span the inner nuclear membrane (15). A fifth NE-specific protein with a putative transmembrane domain, emerin, has been identified in mammals (16–18). Emerin has two short regions of homology to LAP2 (16), but whether it is localized to the inner nuclear membrane has not yet been determined definitively.

All three well characterized integral proteins of the inner nuclear membrane, LAP1 and LAP2 (9) and LBR (11), have been shown to bind to lamins. Through this binding interaction, they could contribute to the attachment of the nuclear lamina to the inner nuclear membrane. Lamins (19–23) as well as LAP2 (9) and LBR (24) bind to chromatin. At least some of these interactions are likely to promote the attachment of chromatin to the NE and higher order chromosome organization in the interphase nucleus. The ability of LAP2 to bind to lamins and chromosomes is regulated by mitotic phosphorylation (9), raising the possibility that the dynamics of this protein during mitosis are closely linked to the processes of NE disassembly and reformation. LBR also is phosphorylated during mitosis (25, 26), but whether this affects its ability to bind to chromatin and lamin is not known.

The question of how integral proteins become targeted to the inner nuclear membrane has been raised by a number of recent studies. During mitosis when the NE is disassembled, LAP1 and LAP2 (27) as well as LBR (28) become dispersed throughout bulk ER membranes, and the NE appears to lose its identity as a distinct membrane system. Conversely these proteins become highly concentrated at the chromosome surfaces during late anaphase, when nuclear membrane reassembly around chromosomes takes place. It has been proposed that segregation of integral membrane proteins to the reforming NE at the end of mitosis is driven by binding interactions at the chromosome surfaces in combination with lateral diffusion through a continuous ER reticulum (6, 28). A similar mechanism may operate during interphase to target newly synthesized integral membrane proteins to the inner nuclear membrane (6, 29, 30). Alternatively, it is conceivable that integral proteins are deliv-
ered to the inner nuclear membrane by some other process that does not involve simple diffusion in the membrane bilayer.

We recently identified a 76-amino acid region in the nucleoplasmic domain of LAP2 which is required for Triton-stable targeting to the NE (10). In this study we have investigated directly whether the NE targeting of LAP2 could be caused by binding interactions at the inner nuclear membrane. For this we mapped the lamin and chromatin binding regions of LAP2. We found that the region of LAP2 which is necessary and sufficient for stable NE targeting coincides with the lamin binding region but is distinct from the region involved in chro-

mosomal binding. We discuss the possibility that binding to laminas could be a major mechanism for targeting integral pro-

teins to the inner nuclear membrane.

EXPERIMENTAL PROCEDURES

Plasmids—For yeast two-hybrid studies (31), members of a set of LAP2 fragments described in (10) were inserted into the BamHI site of the GAL4-DNA binding domain vector pAS2. Full-length cDNAs for lamin B1 (NcoI-AccI fragment), lamin B2 (NcoI fragment), and vimen-
tin (BamHI-EcoRI fragment) were subcloned into the BamHI site of the GAL4 activation domain vector pACT2 using Klenow fill-in and BglII linkers. To construct the Anc1-TM LAP2-lectin fusion, polymerase chain reaction primers (GGC CGA TCC TCA GCG CCC TTC AAA GTA

and grown for 3–5 days at 30 °C. For the liquid method, overnight

streaked to

b

amplify the transmembrane and partial lumenal domains from a

cDNA chain reaction primers (GCG CGA TCC TCA GCG CCC TTC AAA GTA

were generated in the pGEX-2T vector (Pharmacia Biotech Inc.). GST-

LAP2C1 was constructed by subcloning the BamHI ΔC1 fragment as described in Ref. 10 into the BamHI site of pGEX-2T; it encodes amino acids 1–396 of LAP2 fused to the COOH-terminal end of GST. All remaining deletion constructs in this series derive from the parental plasmid, deletion ΔG8, ΔG6, ΔG4, and ΔG3 were constructed by digestion of GST-LAP2C1 with StyI, HindIII, PpuMI-NotI, NotI-HindIII, and NotI, respectively, followed by fill-in with Klenow and re-ligation. Deletion ΔG1 results from digestion of GST-LAP2C1 with XhoI followed by re-ligation, whereas ΔG9 comes from FseI digestion, end-polishing with mung bean nuclease, and re-ligation.

Two-hybrid Assays—A description of the two-hybrid system that was used for this work has been published elsewhere (31). Briefly, Saccha-

romyces cerevisiae strain Y187 was transformed by the procedure of Ref. 34 and grown on complete minimal plates lacking Trp and Leu (~Trp ~Leu medium). Induction of the lacZ reporter gene was monitored by plating on media containing 5-bromo-4-chloro-3-indolyl β-galactopy-

ranoside or direct assay of β-galactosidase activity with O-nitrophenyl-

β-D-galactopyranoside. For platings, freshly grown transformants were streaked to ~Trp ~Leu plates containing 0.04 μg/ml 5-bromo-4-

chloro-3-indolyl β-galactopyranoside and 70 mM sodium phosphate, pH 7.0, and grown for 3–5 days at 30 °C. For the liquid method, overnight cultures grown in ~Trp ~Leu liquid were diluted, grown to A600 = 1.0, and subsequently permeabilized by SDS/chloroform and assayed for β-galactosidase activity.

NE Targeting Assay—5-μg epitope-tagged expression vectors were transfected into HeLa cells by SuperFect transfection (Qiagen, Chatsworth, CA). The localization of chimeric LAP2 proteins was de-
tected by indirect immunofluorescence with anti-HEPA antibody (Babco, Berkeley, CA) as described previously (36). Coverslips were mounted with Slow-Fade anti-fade component (Mol-

cular Probes, Eugene, OR). Slides were viewed on a Zeiss Axioshot microscope configured for epifluorescence illumination, photographed with Kodak Tmax ASA400 film, digitized with a UMAX scanner and prepared for printing on a Kodak Pictography printer using Adobe Photoshop 3.05 software.

Preparation of GST-LAP2 Proteins—GST-LAP2 fusion proteins were expressed in Escherichia coli BL21(pLy83). Overnight cultures were diluted 1:50 into LB medium containing 10 mM magnesium sulfate, 1% glucose, and 100 μg/ml ampicillin at 30 °C. When the culture density reached A600 = 0.5, isopropyl-1-thio-β-D-galactopyranoside was added to 1 mM isopropyl-1-thio-β-D-galactopyranoside and incubation was continued for 45 min. Cells were harvested by centrifugation at 4 °C, resuspended in 1/20 volume of 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 100 mM NaCl and frozen in liquid nitrogen. To prepare extracts, samples were thawed on ice, sonicated twice for 30 s, and cleared by centrifugation at 20,000 × g for 20 min. Extracts were either used directly or purified by binding to glutathione-Sepharose beads (Phar-

macia) for 1 h at 4 °C, followed by washing in alternating cycles of phosphate-buffered saline and phosphate-buffered saline containing 500 mM NaCl, followed by elution in phosphate-buffered saline and 15 mM reduced glutathione buffer. Protein samples were displayed on 12.5% acrylamide-SDS gels and stained with Coomassie Blue to assess the relative GST-LAP2 fusion content.

Chromosome Binding Assay—NRK cells were grown at 37 °C in a humidified incubator containing 5% CO2 atmosphere on coverslips in high glucose Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan UT), 100 units/ml penicillin and streptomycin (Life Technologies, Inc.). To obtain populations enriched in mitotic cells, cultures were grown for 11 h in media containing 2 mM thymidine (Sigma), after which the cells were washed and incubated a further 7 h in the presence of medium lacking thymidine. Samples were removed from the incubator, washed once in a physiologic buffer (TB, Ref. 37), and then incubated for 5 min on ice in TB containing 10 μg/ml digitonin (Calbiochem). After two washes with TB and 10% bovine serum albumin to remove the digitonin, 100 μl of a glutathione affinity-purified GST-LAP2 fusion protein in TB and 10% bovine serum albumin was applied to each coverslip. After 15–20 min incubation at room temperature, samples were fixed immediately by the addition of 2 ml of 4% formaldehyde in TB for 5 min. Samples were subsequently prepared for immunofluorescence microsco-

py as described (27), using goat anti-GST polyclonal serum (1:500, Pharmacia) as primary antibody, and fluorescein isothiocyanate-coupled mouse anti-goat antibody (1:50; Pierce) as secondary antibody. Slides were analyzed as described above for the NE targeting assay.

RESULTS

LAP2 Sequences Required for Lamin Binding—We recently found that a region in the nucleoplasmic domain of LAP2, extending from amino acid 298 to 373, is required for targeting of LAP2 to the NE in a form that is stable to extraction with Triton X-100 (10). Because LAP2 could in principle reach the inner nuclear membrane by diffusion in the membrane bilayer from the peripheral ER (see “Discussion”) and because the nucleoplasmic domain of LAP2 is able to bind to laminas and chromosomes, this suggested that the targeting of LAP2 to the inner nuclear membrane could result from binding to one of these components. To explore this possibility further, we have mapped the regions of LAP2 involved in lamin and chromosome binding to determine whether either of these complexes with the region of LAP2 involved in NE targeting.

We employed the yeast two-hybrid system (31) to identify the lamin binding region of LAP2. The full-length LAP2 open read-


ing frame and a number of deletion variants were cloned into the GAL4-DNA binding domain fusion vector (Fig. 1) and were assayed in yeast in pairwise combinations with GAL4-transcription activation domain fusions containing human lamin B1 or lamin B2. In addition, certain LAP2 constructs were tested for interaction with control fusions containing the cyto-

plasmic intermediate filament vimentin or the yeast SNF4 protein. In this assay system, the binding of LAP2 fragments to lamin B activated a lacZ reporter gene. The activity of this gene was monitored qualitatively by direct plating on 5-bromo-4-

chloro-3-indolyl β-D-galactopyranoside indicator plates for a blue/white color reaction and then was quantitated via direct

assay of soluble yeast extracts for β-galactosidase enzyme activity (35). The results of these experiments are presented in Table I. Strains carrying the full-length LAP2 and either lamin B1 or B2 displayed blue color and high β-galactosidase levels indicative of lacZ reporter induction. Thus, LAP2 binds lamin B in the two-hybrid system. Lamin B protein samples were also biochemically assayed (9). No binding was observed between LAP2 and the vimentin or SNF4 controls in the colony color and β-galactosidase assays, demonstrating the specificity of this interaction. Vimentin provides an especially good control for this interaction because vimentin is a member of the inter-

mediate filament superfamily and has some sequence similar-
between amino acids 296 and 398 (compare terminal border of a sequence required for lamin binding is from the COOH terminus, we determined that the COOH-terminal components (9). In vitro was not observed with deletions are shown. The D NC represents a region of LAP2 (amino acids 298–373) shown previously to be important for stably targeting LAP2 to the NE (10), and the open bar indicates the predicted transmembrane sequence. Panel B, the LAP2/chicken hepatic lectin chimera (ΔNC1-TM) cloned downstream of an HA-tag for expression in cultured cells. The region of LAP2 required for NE targeting was attached to an 81-amino acid fragment of chicken hepatic lectin containing the membrane-spanning domain (open bar) flanked by partial upstream and lumenal sequences (shaded bar).

**TABLE I**

Interactions between LAP2 deletion fragments and lamin B

Two-hybrid interaction assays were carried out with pairwise combinations of the LAP2 constructs shown in Fig. 1 and lamin B1, lamin B2, SNF4 and vimentin as described. β-Galactosidase activity is shown as Miller units of activity in culture extracts and color phenotype (B = blue, W = white) on 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside indicator plates. —, not tested.

| GAL4-DNA binding domain fusion | GAL4-activation domain fusion |
|-------------------------------|-------------------------------|
| FL                            | Lamina B1 | Lamina B2 | SNF4 | Vimentin |
| FL                            | 9.4/B      | 15.3/B     | 0.41/W | 0.32/W |
| ΔC1                           | 10.1/B     | 13.0/B     | 0.38/W | 0.22/W |
| ΔC2                           | 0.13/W     | 0.30/W     | —/W   | —      |
| ΔC3                           | 0.14/W     | 0.75/W     | —/W   | —      |
| ΔN3                           | 14.9/B     | 10.9/B     | —/W   | —      |
| ΔN4                           | 0.15/W     | 0.14/W     | —/W   | —      |
| ΔNC1                          | 2.9/B      | 2.1/B      | —/W   | —      |
| ΔNC2                          | 3.1/B      | 2.9/B      | 0.59/W | —      |
| ΔNC3                          | 9.2/B      | 8.9/B      | —/W   | —      |


Fig. 2. Localization of LAP2 in transfected HeLa cells. Results are from full-length LAP2 (FL) or LAP2 lamin binding region fused to the transmembrane/lumenal domain of chicken hepatic lectin (ΔNC1-TM) as depicted in Fig. 1B. Shown is immunofluorescence staining in the absence (−Triton) or presence (+Triton) of preextraction with Triton.

**FIG. 1. LAP2 deletion constructs used to identify the lamin B binding domain.** Panel A, LAP2 sequences were cloned into pAS2 to generate fusions with the GAL4 DNA binding domain as described. Horizontal lines indicate the LAP2 sequences present in each construct. Full-length (FL), NH2-terminal (ΔN), COOH-terminal (ΔC), and NH2- or COOH-terminal (ΔNC) deletions are shown. The shaded bar represents a region of LAP2 (amino acids 298–373) shown previously to be important for stably targeting LAP2 to the NE (10), and the open bar indicates the predicted transmembrane sequence. Panel B, the LAP2/chicken hepatic lectin chimera (ΔNC1-TM) cloned downstream of an HA-tag for expression in cultured cells. The region of LAP2 required for NE targeting was attached to an 81-amino acid fragment of chicken hepatic lectin containing the membrane-spanning domain (open bar) flanked by partial upstream and lumenal sequences (shaded bar). The Lamin Binding Region of LAP2 Stably Targets a Transmembrane Protein to the NE—Whereas the region of LAP2 between residues 298 and 373 is necessary for stable targeting of transfected deletion mutants of LAP2 to the NE in cultured cells, this segment by itself (which lacks a membrane-spanning sequence) is not sufficient for NE targeting (10). To examine the NE targeting activity of this segment of LAP2 in a more physiological structural context, we fused the LAP2 lamin binding region to a polypeptide containing the transmembrane and partial lumenal domains of chicken hepatic lectin, a type II integral membrane protein like LAP2. Chicken hepatic lectin is localized to the ER and plasma membrane and is not targeted to the NE (32, 38). The lectin fragment used contains amino acids 10–91 of the lectin protein, comprising 15 amino acids of the NH2-terminal sequence, a 30-amino acid hydrophobic segment containing the transmembrane domain, and a 36-amino acid fragment of the lumenal domain. This fusion construct (ΔNC1-TM) and control full-length LAP2 (FL) were expressed as HA-tagged proteins via transfection into cultured HeLa cells, and their localization was determined by immunofluorescent staining with an anti-HA antibody (Fig. 2). The full-length LAP2 was localized in a nuclear rim staining pattern characteristic of NE proteins together with some diffuse cytoplasmic staining (−Triton panel), in agreement with our previous observations (10). Furthermore, the NE association was stable, as indicated by its resistance to extraction in a buffer containing 1% Triton X-100 and 100 mM NaCl (+Triton panel). Localization of ΔNC1-TM, the LAP2 lamin binding segment/chicken hepatic lectin chimera, revealed nuclear rim staining along with a lower level of cytoplasmic staining. The nuclear rim

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| ΔNC1                          | 2.9/B      | 2.1/B      | —/W   | —      |
| ΔNC2                          | 3.1/B      | 2.9/B      | 0.59/W | —      |
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The Lamin Binding Region of LAP2 Stably Targets a Transmembrane Protein to the NE—Whereas the region of LAP2 between residues 298 and 373 is necessary for stable targeting of transfected deletion mutants of LAP2 to the NE in cultured cells, this segment by itself (which lacks a membrane-spanning sequence) is not sufficient for NE targeting (10). To examine the NE targeting activity of this segment of LAP2 in a more physiological structural context, we fused the LAP2 lamin binding region to a polypeptide containing the transmembrane and partial lumenal domains of chicken hepatic lectin, a type II integral membrane protein like LAP2. Chicken hepatic lectin is localized to the ER and plasma membrane and is not targeted to the NE (32, 38). The lectin fragment used contains amino acids 10–91 of the lectin protein, comprising 15 amino acids of the NH2-terminal sequence, a 30-amino acid hydrophobic segment containing the transmembrane domain, and a 36-amino acid fragment of the lumenal domain. This fusion construct (ΔNC1-TM) and control full-length LAP2 (FL) were expressed as HA-tagged proteins via transfection into cultured HeLa cells, and their localization was determined by immunofluorescent staining with an anti-HA antibody (Fig. 2). The full-length LAP2 was localized in a nuclear rim staining pattern characteristic of NE proteins together with some diffuse cytoplasmic staining (−Triton panel), in agreement with our previous observations (10). Furthermore, the NE association was stable, as indicated by its resistance to extraction in a buffer containing 1% Triton X-100 and 100 mM NaCl (+Triton panel). Localization of ΔNC1-TM, the LAP2 lamin binding segment/chicken hepatic lectin chimera, revealed nuclear rim staining along with a lower level of cytoplasmic staining. The nuclear rim
purified on glutathione-Sepharose beads is shown in Fig. 3B. Despite extensive efforts (see “Experimental Procedures”), we were unable to eliminate partial proteolysis of the GST-LAP2 constructs in bacteria. The amount of proteolysis ranged from <5% of total protein (ΔG1, ΔG3 in Fig. 3B) to approximately 70% (ΔG5 in Fig. 3B). Protein amounts in each experiment were normalized such that an equal amount of each intact GST-LAP2 fusion protein was introduced into the binding assay.

The results of this chromosome binding experiment are shown in Fig. 4. A fusion protein containing the complete nucleoplasmic domain of LAP2 (ΔC1) strongly bound to the chromosomes of permeabilized mitotic cells, whereas no binding was detected with GST alone. The mitotic cells in these cell populations were readily detectable by phase-contrast microscopy, which clearly revealed the condensed mitotic chromosomes (e.g. arrowheads in Fig. 4, GST panel). With our experimental conditions, substantial chromosome binding was observed with as little as 15 nM GST-LAP2 fusion protein. No structures in the surrounding interphase cells were detectably labeled with either LAP2 (ΔC1) or GST. In vivo, LAP2 loses a perinuclear distribution and becomes dispersed throughout the cytoplasm during prometaphase by a process that appears to involve LAP2 phosphorylation. It subsequently reassociates with chromosomes in late anaphase and telophase by a mechanism that is apparently dependent on dephosphorylation of LAP2 (9). By contrast, in the present study the binding-competent LAP2 fragments associated with prometaphase and metaphase chromosomes as well as with anaphase and telophase chromosomes. This discrepancy can be explained by the fact that bacterially expressed LAP2 used for our in vitro binding assays lacks the mitotic LAP2 phosphorylation that is involved in dissociation from chromosomes in vivo.

A fusion protein in which the lamin binding domain of LAP2 was deleted (ΔG8) still bound to chromosomes strongly, indicating that lamin and chromosome binding involve separate regions of LAP2. Further deletion of sequences from the COOH-terminal end of the molecule (ΔG6, ΔG3) demonstrated that the NH2-terminal 85 amino acids of LAP2 was sufficient for chromosome association, whereas the first 66 amino acids of LAP2 (ΔG1) was no longer able to bind. In agreement with these results, an internal deletion of amino acids 7–85 (ΔG5) failed to bind to chromosomes, whereas the adjacent deletion (residues 85–194, ΔG4) retained binding activity. It should be noted that the level of chromosome-associated signal in assays involving deletions ΔG6 and ΔG4 was lower than for the other binding constructs, perhaps because of partial misfolding of these deletions. Nevertheless, the binding remained chromosome-specific. The nonbinding constructs GST, ΔG1, and ΔG5 displayed no chromosome-specific labeling even when applied at 20-fold (300 nM) the concentration required for chromosome labeling with the binding-competent constructs (data not shown). Considered together, these data indicate that chromosome binding is specified by the first 85 amino acids of the LAP2 protein, a region distinct from the LAP2 domain required for stable NE targeting and lamin binding.

**DISCUSSION**

**Separate Lamin and Chromatin Binding Regions in LAP2**

Here we have identified the regions of LAP2 involved in lamin and chromosome binding. Using immunofluorescence microscopy to measure the interaction of recombinant LAP2 fragments with mitotic chromosomes, we found that the chromosome binding site of LAP2 resides within residues 1–85. The chromosome binding region of LAP2 determined with our assay is different from a region of LAP2 (amino acids 244–296) which was found to bind single-stranded and double-stranded DNA-cellulose columns (39). Furthermore, we found that the major

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**Fig. 3. LAP2 sequences were cloned into pGEX-2T to generate fusions with GST as described.** Panel A, LAP2 deletion constructs used to identify the chromosome binding domain. Horizontal lines indicate the LAP2 sequences present in each construct. The shaded bar represents a region of the LAP2 protein (amino acids 298–373) shown above to be required for lamin binding and nuclear membrane targeting. Panel B, GST-LAP2 fusion proteins. Equivalent amounts of bacterial extracts from cells containing GST-LAP2 fusion vectors were incubated with glutathione-Sepharose beads for 10 min and washed as described under “Experimental Procedures.” Bound fusion proteins were solubilized in sample buffer, loaded onto a 12.5% SDS gel, and stained with Coomassie Blue. Marks along the left edge of the gel represent a 10-kDa marker ladder; the position of the 50-kDa marker is indicated. When used for binding assays, protein amounts were normalized such that an equal amount of each intact GST-LAP2 fusion protein was used, as determined by Coomassie Blue staining.

staining was stable to Triton extraction, indicating that the chimera was stably targeted to the NE, whereas the cytoplasmic staining was almost entirely removed by this treatment. Thus, the lamin binding region of LAP2 is sufficient to target a heterologous polypeptide containing a transmembrane and partial luminal domain to the NE in a Triton-stable fashion.

**NH2-terminal LAP2 Sequences Specify Chromosome Binding**—To determine whether the chromosome binding region of LAP2 can be distinguished from its major NE targeting/lamin binding domain, we mapped the region of LAP2 which interacts with chromosomes using an in situ binding assay with mitotic cells. For this, cultures of coverslip-attached normal rat kidney cells that were enriched in mitotic populations (see “Experimental Procedures”) were permeabilized by treatment with digitonin and were incubated with recombinant GST fusion proteins containing various regions of LAP2 (Fig. 3). The cells were then fixed and labeled with an anti-GST antibody, and the association of the GST-LAP2 fusions with the chromosomes of mitotic cells was determined by immunofluorescence microscopy. A SDS gel displaying the recombinant GST-LAP2 fusion proteins

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**Fig. 4. Binding interaction of recombinant LAP2 fragments with mitotic chromosomes.** 4A, GST-LAP2 fusion proteins. Equivalent amounts of bacterial extracts from cells containing GST-LAP2 fusion vectors were incubated with glutathione-Sepharose beads for 10 min and washed as described under “Experimental Procedures.” Bound fusion proteins were solubilized in sample buffer, loaded onto a 12.5% SDS gel, and stained with Coomassie Blue. Marks along the left edge of the gel represent a 10-kDa marker ladder; the position of the 50-kDa marker is indicated. When used for binding assays, protein amounts were normalized such that an equal amount of each intact GST-LAP2 fusion protein was used, as determined by Coomassie Blue staining.

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lamin binding region of LAP2 as determined by a yeast two-hybrid assay occurs between residues 298 and 373. Thus, the region of LAP2 which we found previously to be required for Triton-stable targeting to the NE from in vivo transfection studies (10) coincides with the lamin binding site and is distinct from the chromosome binding region. Not only are residues 298–373 required for stable targeting to the NE as demonstrated previously, our present work shows that this segment is sufficient for this targeting when it is attached to the transmembrane region and partial luminal domain of a type II integral protein that normally resides in the ER and has no stable NE association.

We attempted to extend our two-hybrid analysis of the lamin-LAP2 interaction by carrying out binding studies with LAP2 fragments obtained by expression in bacteria and with lamins obtained by in vitro translation or expression in bacteria. We were unable to detect significant lamin-LAP2 binding in any of these experiments. Because specific LAP2-lamin binding was observed previously with LAP2 and lamins purified from rat liver NEs (9), this suggests that the recombinant proteins either were folded improperly or were not postsynthetically modified in a form that allows stable binding.

Because our results show that the chromosome and lamin binding regions of LAP2 are separable, this clearly establishes that the binding of LAP2 to mitotic chromosomes (Ref. 9 and this study) does not occur via lamins that may be bound at low levels to the chromosome surfaces. Furthermore, the presence of separable chromosome and lamin binding domains suggests that LAP2 can potentially bind both substrates simultaneously, such as during nuclear membrane reassembly around chromosomes during late anaphase (discussed in Ref. 27).

The gene encoding LAP2 appears to give rise to at least three different polypeptides in mammalian cells that result from alternative mRNA splicing: LAP2 (thymopoietin β), thymopoietin α, and thymopoietin γ (40). The organization of these proteins, in relation to the domains we have characterized for LAP2, is depicted in Fig. 5. Thymopoietin γ is identical to LAP2 except that it contains an internal deletion equivalent to amino acids 221–328 of LAP2. This deletion removes 31 amino acids from the 76-amino acid region described here as the lamin...
binding/NE targeting domain. Because thymopoietin \( \gamma \) retains a predicted transmembrane domain and appears to be localized to the NE (unpublished data in Ref. 40) it is likely that lamin binding/NE targeting activity is at least partially retained in this protein. The second LAP2-related protein, thymopoietin \( \alpha \), shares only the NH\(_2\)-terminal 187 amino acids with LAP2 and has a large COOH-terminal region that is distinct. It lacks a hydrophobic, potentially membrane-inserted domain as well as the lamin binding/NE targeting region of LAP2 and appears to localize to the interior of the nucleus and not at the NE (unpublished data in Ref. 40).\(^2\) Interestingly, thymopoietins \( \alpha \) and \( \gamma \) both contain the LAP2 chromosome binding domain, suggesting that each of these proteins is capable of a chromatin interaction. At the present time, the molecular characteristics and functions of this chromatin binding by the LAP2/thymopoietin \( \alpha \) are unknown. It should be noted that additional classes of cDNAs derived from the LAP2 gene have been found in mouse (41). However, these novel cDNAs could not be identified in human cells (41), and we have been able to detect only LAP2 and thymopoietins \( \alpha \) and \( \gamma \) in rat liver and a variety of cultured rat cells with anti-LAP2 polyclonal sera.\(^2\)

![Fig. 5. Predicted domain structure of the LAP2 protein family.](http://www.jbc.org/) Schematic diagrams of LAP2 (thymopoietin \( \beta \)) and thymopoietins \( \alpha \) and \( \gamma \) are shown. The LAP2 domains for transmembrane insertion (single line), lamin binding/NE targeting (shaded bar), and chromosome binding (hatched bar) are also shown in thymopoietins \( \alpha \) and \( \gamma \) if the identical sequences occur. A portion of the lamin binding/nuclear membrane targeting domain is contained in the LAP2 sequences deleted from thymopoietin \( \gamma \). Thymopoietin \( \alpha \) from amino acids 189–673 is unrelated to LAP2 and lacks a predicted hydrophobic transmembrane region.

**Functions of Lamin and Chromatin Binding by LAP2**—The interaction of LAP2 with nuclear lamins could play a role in the organization of the lamina (e.g. attachment to the NE) and its dynamics during interphase. In support of this possibility, we found recently that microinjection of the 76-amino acid lamin binding segment of LAP2 into mitotic or interphase mammalian cells strongly inhibits nuclear growth, but not NE assembly, probably by interfering with lamina dynamics (42). This raises the possibility that the interaction of LAP2 with the lamins could, among other things, serve to regulate lamina growth negatively. In a related function, the association of LAP2 with both lamins and chromatin could contribute to the reassembly of nuclear membranes and lamins around the chromosomal surfaces at the end of mitosis. Nevertheless, it is plausible that NE reassembly is a highly cooperative process involving lamins and several integral proteins in addition to LAP2 (discussed in Refs. 27 and 43). Finally, we suggest that the binding interactions of LAP2 could have a role in biogenesis of the NE during interphase, by serving to target LAP2 to the inner nuclear membrane.

Because the region of LAP2 which is necessary and sufficient for stable targeting to the NE coincides with the lamin binding region but not the chromatin binding segment, this suggests that the targeting of LAP2 to the inner nuclear membrane is based primarily on lamin binding. Nevertheless, it remains possible that the association of LAP2 with chromatin contributes some (albeit weaker) NE targeting activity. Our previous transfection studies showed that an LAP2 mutant containing an internal 104-amino acid deletion that included the 76-residue lamin binding region we have identified accumulated at the NE, although the NE-associated protein was not stable to Triton extraction (10). In this case the NE targeting could have been caused by either the NH\(_2\)-terminal chromosome binding domain of LAP2 or a hypothetical secondary lamin binding region of LAP2 which flanks the core lamin binding segment. In the case of the LBR protein, the NE targeting region has been shown to reside in the first 200 amino acids of the nucleoplasmic domain and first transmembrane segment, a region that also contains binding activities for lamins and chromosomes (38, 44, 45). However, the role of these individual binding activities in NE targeting has not yet been examined.

How could LAP2 be targeted to the inner nuclear membrane by binding interactions at the lamina, considering the structural properties of the NE? Although the inner and outer nuclear membranes are in direct continuity via the pore membrane, the pore complex could potentially present a topological barrier to the movement of LAP2 and other integral proteins from their site of synthesis in the peripheral ER to the inner nuclear membrane. However, structural studies have indicated that ~10-nm-diameter channels occur at the periphery of the pore complex immediately adjacent to the pore membrane (46). In principle these channels could allow the free diffusion of integral proteins with sufficiently small nucleoplasmic domains around the pore membrane between the outer and inner nuclear membranes. With such a bidirectional diffusional exchange, proteins could become trapped at the inner nuclear membrane by virtue of binding interactions with the nuclear lamina (6). Consistent with this model, another integral protein of the inner nuclear membrane, LAP1C, has been shown to be capable of rapid exchange between the two nuclei of heterokaryons, presumably by diffusing through a common ER network (47). This suggests that LAP1C continuously undergoes bidirectional movement between the peripheral ER and the inner nuclear membrane, even though it is highly concentrated in the latter membrane at steady state. No evidence exists at present to support an alternative targeting mechanism involving active, unidirectional movement of proteins from the peripheral ER to the inner nuclear membrane.

We believe that a general mechanism for the targeting of...
integral proteins to the inner nuclear membrane is likely to involve binding interactions at the NE. Both LAP1 and LAP2 are stably anchored to the nuclear lamina, as indicated by their resistance to extraction from the lamina by detergent treatments (7). Moreover, recent measurements of the diffusion dynamics of a fusion protein containing LBR in living cells indicated that the motion of this protein at the inner nuclear membrane was strongly restricted (28), also indicative of strong binding interactions at the inner nuclear membrane. The fact that all three well characterized integral membrane proteins of the inner nuclear membrane interact with lamins suggests that lamin binding in particular may function to target proteins to the inner nuclear membrane. This model is made particularly appealing by the fact that lamins constitute extremely abundant inner membrane proteins and appear to be associated with the inner nuclear membrane in virtually all higher eukaryotic cells. A prediction of this model is that dissociation of lamins from the NE by microinjection of a dominant negative lamin mutant (48) or by other means would result in the rapid redistribution of inner membrane proteins to the peripheral ER during interphase.

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