The First Membrane Spanning Region of the Lamin B Receptor Is Sufficient for Sorting to the Inner Nuclear Membrane

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Abstract. The lamin B receptor (LBR) is a polytopic integral membrane protein localized exclusively in the inner nuclear membrane domain of the nuclear envelope. Its cDNA deduced primary structure consists of a highly charged amino-terminal domain of 205 residues that faces the nucleoplasm followed by a hydrophobic domain with eight potential transmembrane segments. To identify determinants that sort LBR from its site of integration (RER and outer nuclear membrane) to the inner nuclear membrane, we prepared full-length, truncated, and chimeric cDNA constructs of chick LBR, transfected these into mammalian cells and detected the expressed protein by immunofluorescence microscopy using appropriate antibodies. Surprisingly, we found that the determinants for sorting of LBR to the inner nuclear membrane reside in a region comprising its first transmembrane sequence plus flanking residues on either side. The other transmembrane regions as well as the nucleoplasmic domain are not required for sorting. We propose that the first transmembrane segment of LBR interacts specifically with another transmembrane segment and consider several mechanisms by which such specific interaction could result in sorting to the inner nuclear membrane.

USING solution and solid state binding assays, isolated lamin B from turkey nuclei has previously been shown to bind to an integral membrane protein of the nuclear envelope termed the lamin B receptor (LBR)[1] or p58 (because of its apparent molecular weight in SDS-PAGE) (Worman et al., 1988). Cell fractionation (Worman et al., 1990) and immunoelectron microscopy (Yuan, J., H. J. Worman, and G. Blobel, unpublished results) revealed that LBR is exclusively localized in the inner nuclear membrane. LBR's cDNA deduced primary structure showed a protein of 637 amino acid residues with two major domains: an amino terminal hydrophilic and basic domain of 205 residues followed by a predominantly hydrophobic region with eight putative transmembrane segments (Worman et al., 1990). The amino terminal domain of 205 residues is likely located on the nucleoplasmic (rather than the cisternal) side of the inner membrane because it contains all of LBR's consensus sites for various kinases (Feramisco et al., 1980; Moreno and Nurse, 1990) and LBR has been shown to be a phosphoprotein (Applebaum et al., 1990). The amino terminal nucleoplasmic domain of LBR also contains SPXX motifs that are characteristic of nucleic acid binding proteins (Suzuki, 1989). It is not known, however, whether LBR interacts with nucleic acids.

LBR's binding site for lamin B has not yet been identified. During mitosis lamin B appears to be dissociated from LBR (Chaudhary, N., G. Blobel, and J. C. Courvalin, unpublished results) in conjunction with the phosphorylation and dephosphorylation at specific sites of the LBR nucleoplasmic domain (Courvalin et al., 1992). Because of its topology this domain is a candidate for containing LBR's lamin B binding site, which in turn is a candidate for being a determinant for sorting LBR to the inner nuclear membrane. Like other membrane proteins that are newly integrated into the RER membrane system (Bergmann and Singer, 1983; Torrisi and Bonatti, 1985; Torrisi et al., 1987; Powell and Burke, 1990) LBR would have access to the inner nuclear membrane through lateral diffusion via the pore membrane domain of the nuclear envelope. Once there, it could simply be tethered by binding to lamin B. Using in vivo expression of full-length, truncated, and chimeric LBR cDNAs we found, however, that it is not the nucleoplasmic domain but the region of the first transmembrane segment that constitutes the primary determinant for sorting of LBR to the inner nuclear membrane.

1. *Abbreviations used in this paper*: HA, hemagglutinin; LBR, lamin B receptor; P, pellet; PCR, polymerase chain reaction; PL, prolactin; pPL, preprolactin; RuBPCase, ribulose-1,5-bisphosphate carboxylase; S, supernatant; SSRα, signal sequence receptor α.

Materials and Methods

DNA Constructs

All constructs were made using standard cloning procedures (Maniatis et al., 1989).

p58cDNA. A full-length cDNA consisting of the 5' untranslated region, codons 1 through 637, and the 3' untranslated region was generated from two overlapping clones, DJ-5 and M-3, described previously (Worman et al., 1990), by ligation at a common internal, unique AccI site and cloned into the EcoRI site of Bluescript SK− (Stratagene, La Jolla, CA).

pSVX. A fragment containing a translational stop codon in all three read-

ing frames was generated using complementary oligonucleotides, 5'-TCGATGACCTATGAACTATGAGCTA-3' and 5'-TCGATAGCTCATAGTTCATAGGTCA-3', and inserted into the XhoI site in the polylinker of the SV-40–based expression vector, pSVK3 (Pharmacia Fine Chemicals, Piscataway, NJ). All truncated constructs were expressed in the pSVX vector to ensure that there was a stop codon at the end of the open reading frame.

p58ΔTM2-8, Including Insertion of the HA Tag. p58cDNA was digested with BsmAI, blunt ended with DNA polymerase, Klenow fragment, and digested with EcoRI. The resulting fragment (containing the 5' untranslated region and codons 1 through 246) was cloned into the EcoRI and SmaI sites of the pSVX polylinker in the correct orientation with respect to the SV-40 early promoter. Subsequently, the HA tag was inserted in frame into this construct. A fragment containing the nine codons of the HA epitope tag (Wilson et al., 1984) and flanking SpeI sites was generated by two complementary oligonucleotides 5'-CTAGTTACCCATACGATGTTCCAGATTACGCTG-3' and 5-CTAGCAGCGTAATCTGGAACATCGTATGGGTAA-3' (HA codons are underlined) and inserted into the unique SpeI site beginning at codon 28 of p58. Insertion of the oligonucleotide regenerated the site 5' but not 3' to the tag and thus, insertion of a single HA tag in the correct orientation was confirmed by restriction enzyme digest analysis. The resulting construct, p58ΔTM2-8, contained p58 codons 1 through 29, nine codons of the HA tag, a serine codon (AGT) introduction by the cloning, and p58 codons 30 through 246. The organization of the HA tag was identical in all constructs. p58ΔTM2-8 contains p58 amino acids 1–246.

p58ΔTM1-8. p58cDNA was digested with HinfI, blunt ended with DNA polymerase, Klenow fragment, and digested with EcoRI. The resulting fragment (containing the 5' untranslated region and codons 1 through 206) was cloned into the EcoRI and SmaI sites of the pSVX polylinker in the correct orientation with respect to the SV-40 early promoter and the HA tag was inserted exactly as described for p58ΔTM2-8. p58ΔTM1-8 contains p58 amino acids 1–206.

p58FL. The full-length cDNA was isolated from p58cDNA by digestion of the polylinker restriction sites with SmaI (5' to the cDNA) and XhoI (3' to the cDNA) and cloned into the SmaI and XhoI sites of the pSVK3 polylinker in the correct orientation with respect to the SV-40 early promoter. To generate a full-length HA-tagged construct, a KpnI–SacI fragment (containing codons 45 through 637 and the 3' untranslated region) was isolated and used to replace the KpnI–SacI fragment (containing amino acids 45 through 206) of p58ΔTM1-8. p58FL contains p58 amino acids 1–637.

p58ΔN. A fragment containing codons 190 through 637 and the 3' untranslated region was amplified from p58FL by the polymerase chain reaction (PCR) using a 5' PCR primer, 5'-GAGAATTCGGTACCCCGGAGAAACCATCATCA-3' (KpnI site is underlined) and 3' PCR primer, 5'-CTGAATTCTCTAGAAGGTCATAGACAGTCAGT-3' (XbaI site is underlined). The resulting fragment was digested with KpnI and XbaI and used to replace the KpnI–XbaI fragment (containing codons 45 through 206) of p58ΔTM1-8. p58ΔN contains p58 amino acids 1–45 + 190–637.

p58ΔTM1+2. This plasmid was derived from an intermediate construct, p58ΔN,TM1+2. A fragment containing codons 280 through 637 and the 3' untranslated region was amplified from p58FL by PCR using a 5' PCR primer, 5'-GAGAATTCGGTACCAATCCAAGGAAGCTGCAG-3' (KpnI site is underlined) and 3' PCR primer, 5'-CTGAATTCTCTAGAAGGTCATAGACAGTCAGT-3' (XbaI site is underlined). The resulting fragment was digested with KpnI and XbaI and used to replace the KpnI–XbaI fragment (containing codons 45 through 206) of p58ΔTM1-8. p58ΔN,TM1+2 contains p58 amino acids 1–45 + 280–637. Next, a fragment containing the complete amino terminal domain was amplified from p58ΔTM2-8 by PCR using a 5' PCR primer, 5'-GGGAATTCGGCACGAGCTGAGGAGCA-3' and 3' PCR primer 5'-GGGGTACCTCTTCCACCAAATTCTAG-3' (KpnI site is underlined). The resulting fragment was digested with KpnI and the fragment containing codons 45 through 205 was ligated into the unique KpnI site of p58ΔN,TM1+2. The correct orientation was determined by restriction digest analysis. p58ΔTM1+2 contains amino acids 1–205, followed by a glycine and threonine (introduced by the cloning), followed by amino acids 280–637.

Ru-p58. The starting point for the cloning was a plasmid, pET8c/S (a gift from Dr. Danny Schnell), which contained the DNA sequence encoding the mature small subunit of ribulose-1,5-bisphosphate carboxylase (RuBPCase) cloned into the prokaryotic expression vector pET8c (Rosenberg et al., 1987). pET8c/S was digested with XbaI, which cuts 5' to the gene, blunt ended with DNA polymerase, Klenow fragment, and digested with KpnI, a unique site within the RuBPCase gene. This fragment (containing the first 76 codons of the mature subunit of RuBPCase) was used to replace the 5'-untranslated region and codons 1 through 45 and p58ΔN (including the HA tag) by cloning into the large fragment of p58ΔN produced by digestion with EcoRI, blunt end formation with DNA polymerase, Klenow fragment and digestion with KpnI. Ru-p58 contains p58 amino acids 190–637.

βgal-TM1. pCH110 (Pharmacia Fine Chemicals), a β-galactosidase expression plasmid, was digested with ScaI and EcoRI and the resulting large fragment (containing the 5' half of the ampicillin gene, the SV-40 early promoter, and the gene encoding a lac Z fusion protein), was ligated to the ScaI–EcoRI fragment of pSVX containing the 3' half of the ampicillin gene and the polylinker. The resulting plasmid contained the lac Z fusion (minus the last 16 amino acids from the carboxy end) fused to the polylinker in pSVX. Next, a fragment containing codons 201 through 246 was amplified from p58ΔTM2-8 by PCR using 5' PCR primer, 5'-GAGAATTCGGTGGAAGATTCGGGACC-3' (EcoRI site is underlined) and 3' PCR primer, 5'-GTCGACTCTAGAGGATCC-3' (XbaI site is underlined). The resulting fragment was digested with EcoRI and XbaI and cloned into the EcoRI and XbaI sites of the polylinker in the pCH110-derived vector, 3' to and in frame with the lac Z gene. βgal-TM1 contains p58 amino acids 201–246.

In Vitro Transcription and Translation

The specific mRNA encoding p58 was prepared by T3 RNA polymerase in vitro transcription of p58cDNA linearized with XhoI. mRNA encoding bovine preprolactin was prepared by T7 RNA polymerase in vitro transcription of pGEMBP1 (Connolly and Gilmore, 1986) linearized with EcorI. The mRNAs were used to program in vitro translation reactions using the reticulocyte lysate system (Promega, Madison, WI) as described previously (Nicchitta and Blobel, 1989). Before analysis of translation products, reactions were fractionated by ammonium sulfate precipitation according to Nicchitta and Blobel (1990). The pellets were dissolved in sample buffer containing 0.5 M Tris base, 5% SDS, and 50 mM DTT, incubated for 30 min at 55°C, followed by bath sonication for 60 s. Samples were fractionated on 12.5% SDS–polyacrylamide gels according to the procedure of Laemmli (1970), enhanced with Enlightning (Dupont New England Nuclear, Boston, MA), and autoradiographed.

For immunoprecipitation, the in vitro translation reactions were diluted fivefold with dilution buffer (50 mM Tris, pH 8.0, 1% NP-40, 150 mM NaCl, 1 mM PMSF) and incubated with 5 μl of control guinea pig serum or anti-p58 guinea pig serum (Worman et al., 1988). The immune complexes were precipitated with protein A-Sepharose (Pharmacia Fine Chemicals), washed three times with dilution buffer, and then analyzed by SDS-PAGE as described above.

Membrane Integration

In vitro translation was performed in the presence of rough microsomal membranes as described (Nicchitta and Blobel, 1989). After translation, reactions were chilled on ice and subjected to sedimentation analysis through sucrose step cushions according to (Nicchitta and Blobel, 1990). Where indicated, translation reactions were extracted with 9 Vol of 6 M urea or 9 Vol of 0.1 M Na_2CO_3, pH 11.5, for 10 min on ice, and then fractionated by centrifugation as described (Gilmore and Blobel, 1985). Supernatants and pellets were processed and analyzed by SDS-PAGE as described above.

Peptide Antibodies

A peptide containing amino acids 2–17 of chick p58 (Worman et al., 1990) was coupled to keyhole limpet hemocyanin (Calbiochem Behring Corp., La Jolla, CA) through an additional carboxy-terminal cysteine with maleimido *bis* succinimide (MBS; Pierce Chemical Co., Rockford, IL) as described (Green et al., 1982) and used for the preparation of rabbit polyclonal antisera as described (Harlow and Lane, 1988). The p58 synthetic peptide was coupled to SulfoLink coupling gel (Pierce Chemical Co.) according to the supplier's protocol. Anti-p58 IgG were then affinity purified by passing crude serum over the peptide resin, washing with 0.5 M NaCl, eluting with 0.1 M glycine, pH 2.5, and dialyzing against PBS.

Cells and Transfection

COS-1 cells were maintained in DME (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Gibco Laboratories). Cells were transfected using the calcium phosphate transfection protocol of Chen and Okyama, (1987). For immunofluorescence experiments, COS-1 cells were plated on coverslips in 16-mm wells (~50% confluent) and transfected with 1 μg of supercoiled DNA which was purified on Qiagen columns (Qiagen, Chatsworth, CA) according to the supplier's protocol. Cells were analyzed by indirect immunofluorescence microscopy 24–40 h after transfection. The number of cells expressing the transfected protein ranged from 5% to 40% depending on the construct.

Indirect Immunofluorescence Microscopy

All manipulations were done at room temperature. Cells were washed with PBS after each step of fixation and antibody incubation. Antibodies were diluted in 3% BSA/PBS. Transfected cells on coverslips were fixed for 3 min in 3.7% formaldehyde and permeabilized for 5 min with 0.5% Triton X-100. Cells were blocked for 15 min in 3% BSA/PBS, followed by incubation for 60 min with one of the following primary antibodies: affinity-purified rabbit anti-p58 IgG (10 μg/ml; described above); rabbit antiserum raised against the small subunit of RuBPCase (1:100; a gift from Dr. Debkumar Pain), or fractionated ascites fluid developed in mouse against β-galactosidase (1:1000; Sigma Immunochemicals, St. Louis, MO), followed by a 15-min incubation with the appropriate second antibody; goat anti-mouse or goat anti-rabbit IgG-FITC (1:50; Cappel, Durham, NC). To measure the endogenous COS cell p58, human autoantibody (1:100; Courvalin et al., 1990), followed by anti-human IgG-FITC (1:50; Bio-Rad Laboratories, Richmond, CA) was used. For the double immunofluorescence experiment, the two primary antibodies, affinity-purified anti-SSRα (4 μg/ml; Migliaccio et al., 1992) and a mAb to the HA tag (1:200; Berkeley Antibody Company, Richmond, CA) were incubated concomitantly as were the secondary antibodies, donkey anti-mouse IgG-FITC and donkey anti-rabbit IgG-Texas red (1:50; Jackson ImmunoResearch Labs, Inc., West Grove, PA). Coverslips were mounted in ρ-phenylenediamine solution (1 mg/ml) in 90% glycerol in PBS, pH 8.0 (Johnson and Nogueria Araujo, 1981). Cells were viewed under an Axiophot (Carl Zeiss, Oberkochen, Germany) and photographed with T-MAX 400 Kodak film (Eastman Kodak Co., Rochester, NY).

The nuclear envelope staining pattern displayed in each figure is typical for the majority of cells (70–90%) expressing the transfected gene. A minority of the expressing cells in each experiment (10–20%) produced unusually high levels of the transfected protein resulting in an obscuring of the exclusive rim stain. In these cases the protein appeared in the RER, Golgi complex, and in some cases on the cell surface. Overexpression and the resulting loss in the fidelity of localization has been discussed by others using similar transfection systems (Li and Bingham, 1991; Munro, 1991). This pattern correlates with extremely high levels of expression and is therefore most apparent at late times after transfection (48–72 h). Thus, we restricted our analysis to 24–40 h after transfection.

Results

In Vitro Integration of LBR into Rough Microsomes

The primary structure of LBR (depicted schematically in Fig. 1 *A*) was previously derived from overlapping cDNA clones. We constructed a full-length cDNA (see Materials and Methods), expressed the protein in a cell-free reticulocyte lysate system (Fig. 1 *B*), and immunoprecipitated the protein with the previously characterized guinea pig antibodies against turkey LBR (Worman et al., 1988) (Fig. 1 *C*). Like its in vivo synthesized counterpart the in vitro expressed LBR migrated faster than expected, as a protein of 58 kD rather than the expected 73 kD.

Amino terminal protein sequencing data and cDNA deduced primary structure had established that LBR is synthesized without a cleavable amino terminal signal sequence (Worman et al., 1990). Its integration into the membrane therefore is likely to proceed by its transmembrane segments serving as internal signal and stop transfer sequences (Blobel, 1980; Friedlander and Blobel, 1985) leaving the 205 residue long hydrophilic domain untranslocated on the cytoplasmic side of the ER membrane. In vitro expressed LBR could be integrated into rough microsomal membranes (Fig. 2). In the absence of membranes (−MEM), both LBR (p58) as well as a secretory protein, preprolactin (pPL), remained in the supernatant (S) fraction, whereas in the presence of membrane (+MEM) most p58 and processed prolactin (PL) cosedimented with the membrane in the pellet (P) fraction (Fig. 2 *A*). LBR (p58) was integrated into membranes as it continued to sediment in the pellet fraction after

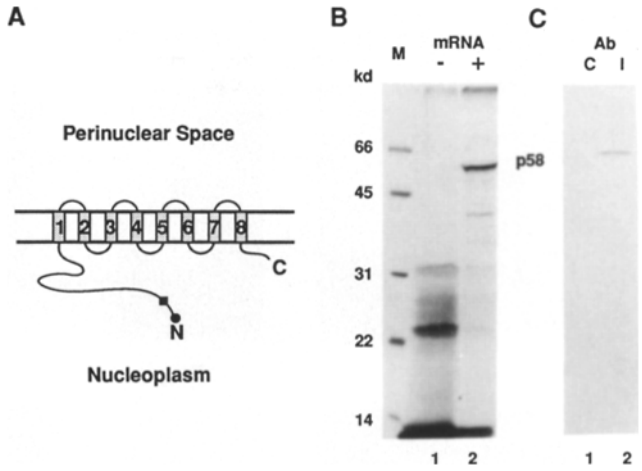

Figure 1. (*A*) Topological model of LBR based upon the predicted amino acid sequence. The rectangles numbered 1 through 8 show the potential membrane spanning segments. The circle and square indicate the location of the p58 peptide against which the antibody was raised and the inserted HA epitope, respectively. (*B*) In vitro translation of LBR. In vitro translation in a reticulocyte lysate system was performed in the absence (lane *1*) or presence (lane *2*) of p58 mRNA. Molecular weight markers (*M*) are indicated. (*C*) Immunoprecipitation of LBR. p58 mRNA was translated in vitro in a reticulocyte lysate system and the reaction products were immunoprecipitated with control (*C*) guinea pig serum (lane *1*) or immune (*I*) guinea pig anti-p58 serum (lane *2*). The protein products of the reactions in *B* and *C* were processed as described in Materials and Methods, resolved on 12.5% SDS-PAGE, and detected by autoradiography.

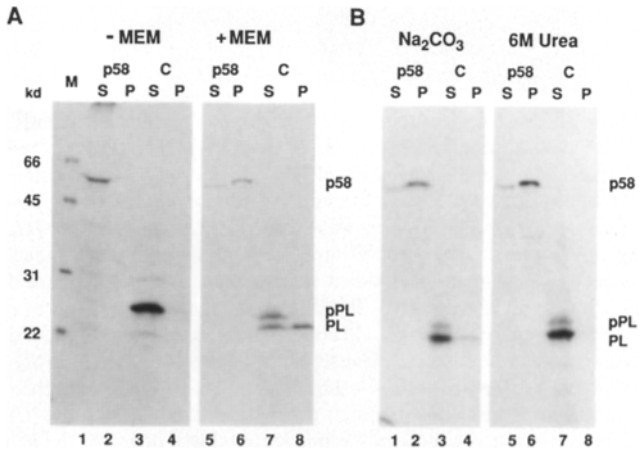

Figure 2. (*A*) Membrane association of LBR. In vitro translation in a reticulocyte lysate system containing p58 mRNA (*p58*) or preprolactin (*pPL*) mRNA (*C*) was performed in the absence (*−MEM*) or presence (*+MEM*) of microsomal membranes. Samples were subjected to centrifugation and separated into supernatant (*S*) and pellet (*P*) fractions as described in Materials and Methods. Molecular weight markers (*M*) are indicated. (*B*) Membrane integration of LBR. In vitro translation in a reticulocyte lysate system containing p58 mRNA (*p58*) or pPL mRNA (*C*) was performed in the presence of microsomal membranes. The reactions were extracted with 0.1 M Na_2CO_3, pH 11.5, or 6 M urea, and soluble proteins (*S*) were separated from membrane integrated proteins (*P*) by centrifugation as described in Materials and Methods. Protein samples in *A* and *B* were processed as described in Materials and Methods, resolved on 12.5% SDS-PAGE and detected by autoradiography. pPL and PL indicate the position of preprolactin and mature prolactin, respectively.

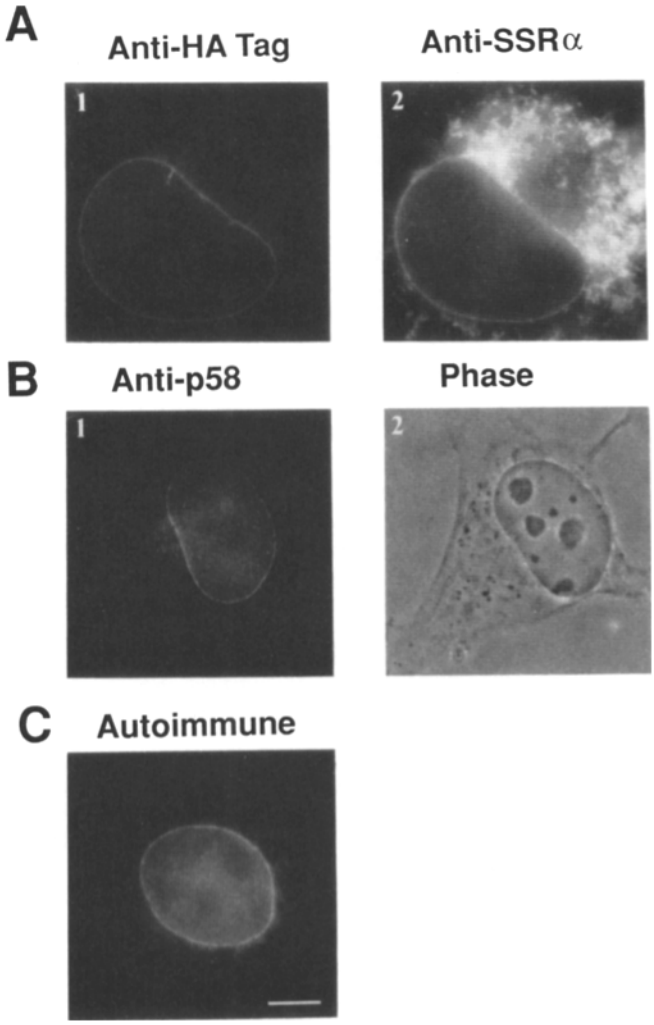

Figure 3. LBR localizes to the nuclear envelope in transfected cells. COS-1 cells transfected with p58FL *A* and *B* or mock transfected (*C*), were fixed and permeabilized 40 h (*A* and *C*) or 24 h (*B*) after transfection and processed for indirect immunofluorescence microscopy. (*A*) Double staining was performed with mouse anti-HA tag mAbs (panel *1*) and rabbit anti-SSRα anti-peptide antibodies (panel *2*). Secondary antibodies were anti-mouse IgG-FITC and anti-rabbit IgG-Texas red. Panel *1* was photographed with a fluorescein filter and panel *2* shows the same cell photographed with a rhodamine filter. (*B*) Cells were stained with rabbit anti-p58 antibodies, followed by anti-rabbit IgG-FITC and photographed with a fluorescein filter (panel *1*) or phase contrast (panel *2*). (*C*) Cells were stained with autoimmune serum that recognizes the endogenous COS cell LBR, followed by anti-human IgG-FITC. Bar, 10 μm.

treatment with Na_2CO_3 at pH 11.5 or 6 M urea, both of which extracted the translocated (processed) secretory protein (Fig. 2 *B*).

Nuclear Rim Localization of Chick LBR in COS Cells

To detect chick LBR expressed in COS cells, an epitope-tagged chick LBR cDNA was constructed (see Materials and Methods) that contained a nine amino acid epitope of the influenza virus hemagglutinin (HA) inserted after Ser 29 of p58. The epitope-tagged cDNA was cloned into an SV-40 expression vector and the construct (p58FL) was introduced into COS cells by calcium phosphate–mediated transfection. Localization of the protein was monitored by immunofluorescence microscopy using anti-HA mAbs. As shown in Fig. 3 *A, 1*, the protein was localized predominantly at the nuclear rim. In contrast, when the same cell was stained with antibodies to an endogenous integral membrane protein of the ER, known as signal sequence receptor α (SSRα) (Wiedmann et al., 1987), there was nuclear rim staining and diffuse cytoplasmic staining characteristic for an ER protein (Fig. 3 *A, 2*). As the ER and the nuclear envelope are one continuous membrane system that is accessible by lateral diffusion to all membrane proteins targeted to and integrated into the ER, the exclusive nuclear rim staining of HA-tagged p58 can be considered to represent correct sorting to the inner nuclear membrane. If the HA-tagged p58 were specifically sorted to the outer nuclear membrane it should also be located in the ER, and like SSRα, should yield cytoplasmic staining which it did not.

The HA-tagged protein could also be detected using anti-p58 antibodies (Fig. 3 *B, 1*) directed against a synthetic peptide (amino acid residues 2 to 17) of chick LBR. The antipeptide antibodies did not react with the endogenous COS cell LBR in immunofluorescence microscopy or on immunoblots (data not shown) and thus, HA tagging was not necessary to distinguish foreign from endogenous LBR. However, for unknown reasons LBR constructs lacking the HA tag, while producing a typical rim staining pattern, were expressed at low frequency and at very low levels (data not shown). COS cells do synthesize their own LBR that can be readily detected using a previously characterized human autoimmune serum (Courvalin et al., 1990) (Fig. 3 *C*). Again, a characteristic nuclear rim staining was obtained (Fig. 3 *C*) indistinguishable from that of HA-tagged chick LBR (Fig. 3, *A* and *B*).

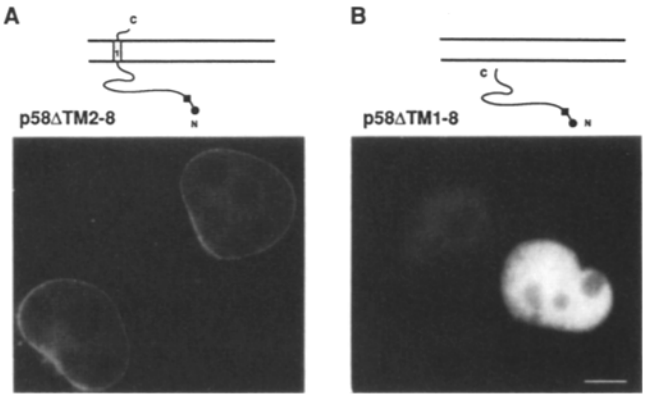

Figure 4. The first of eight transmembrane domains is sufficient to target LBR to the nuclear envelope. COS-1 cells transfected with p58ΔTM2–8 (*A*) or p58ΔTM1–8 (*B*) were fixed and permeabilized 40 h after transfection and processed for indirect immunofluorescence by staining with rabbit anti-p58 antibodies followed by anti-rabbit IgG-FITC. Bar, 10 μm.

Localization of Carboxy-terminally Truncated LBR

As the nucleoplasmic domain was a prime candidate for being the determinant for sorting to the inner nuclear membrane, we made two carboxy-terminally truncated LBR constructs. One of these was truncated at residue 246. It contained the nucleoplasmic domain, the first transmembrane segment and 20 adjacent residues but lacked the remaining seven transmembrane segments and we therefore refer to it as p58ΔTM2-8 (see cartoon in Fig. 4 *A*). Expression

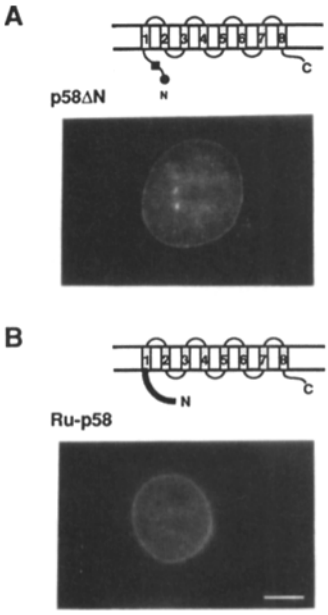

Figure 5. The amino terminal domain of LBR is not required for targeting to the nuclear envelope. COS-1 cells transfected with p58ΔN (*A*) or Ru-p58 (*B*) were fixed and permeabilized 40 h (*A*) or 24 h (*B*) after transfection. Cells were processed for indirect immunofluorescence microscopy by staining with rabbit anti-p58 antibodies (*A*) or rabbit antibodies against the small subunit of RuBPCase (*B*), followed by anti-rabbit IgG-FITC. Bar, 10 μm.

in COS cells yielded the characteristic nuclear rim staining (Fig. 4 *A*) indistinguishable from that of the full-length LBR (Fig. 3, *A* and *B*). These data indicated that the carboxy-terminal part of LBR including seven of its eight transmembrane segments are not required for sorting to the inner nuclear membrane.

The other carboxy-terminally truncated LBR construct (truncated at residue 206) contained only the nucleoplasmic domain of LBR, lacked all of the eight transmembrane domains and therefore was referred to as p58ΔTM1-8 (see cartoon in Fig. 4 *B*). Interestingly this protein localized exclusively to the nucleus, excluding nucleoli, with no detectable enrichment at the nuclear rim (Fig. 4 *B*). The exclusive nuclear localization suggests that this protein enters the nucleus and perhaps by virtue of an affinity for a chromatin component is retained there (see Discussion).

The data of the carboxy-terminal truncation experiments suggested that the determinant for sorting to the inner nuclear membrane is located in the first third of the molecule comprising LBR's nucleoplasmic domain and the first transmembrane region.

The Nucleoplasmic Domain Is Not Required for Sorting

To further sublocalize LBR's sorting determinant we prepared an amino terminally truncated LBR (p58ΔN) in which residues 46 to 190 of the 205 residue long nucleoplasmic domain were deleted. We found nuclear rim staining (Fig. 5 *A*) indistinguishable from that of full-length LBR, indicating that much of the nucleoplasmic domain is dispensable for sorting.

Next we made a chimeric construct (Ru-p58) in which the nucleoplasmic domain, up to residue 190, was replaced by the small subunit of RuBPCase, a protein that is located in chloroplasts. Immunolocalization showed nuclear rim staining characteristic of LBR (Fig. 5 *B*). These data indicated that substitution of the nucleoplasmic domain by a heterologous protein still yields sorting to the inner nuclear membrane. These data suggest that the nucleoplasmic domain is not required for sorting to the inner nuclear membrane.

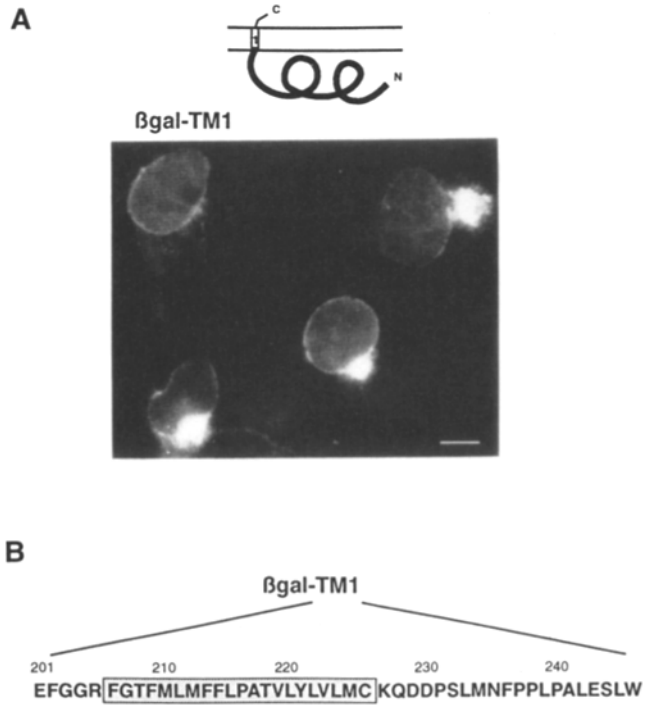

Figure 6. The first transmembrane region of LBR sorts β-galactosidase to the inner nuclear membrane. (*A*) COS-1 cells transfected with βgal-TM1 (*B*) were fixed and permeabilized 24 h after transfection and processed for indirect immunofluorescence microscopy by staining with mouse anti–β-galactosidase antibodies, followed by anti-mouse IgG-FITC. (*B*) shows the amino acid sequence encoded by the chick p58 sequence contained in the βgal-TM1 construct. The stippled box encloses the predicted membrane spanning segment. Bar, 10 μm.

The First Transmembrane Region Is Sufficient for Sorting

So far, the data have localized LBR's sorting determinant to the first transmembrane region. To test whether this region is sufficient we made a chimeric construct in which the bacterial protein, β-galactosidase, replacing the nucleoplasmic domain, was linked to LBR's first transmembrane region comprising residues 201-246 of LBR (Fig. 6). Immunolocalization of this protein (βgal-TM1) yielded nuclear rim staining (Fig. 6 *A*). In addition, there was accumulation in a distinct region in the cytoplasm, which did not colocalize with the Golgi complex or the RER (based upon double immunofluorescence using wheat germ agglutinin or anti-SSRα antibody, respectively), data not shown. The additional cytoplasmic staining may reflect an accumulation of unassembled or aggregated fusion protein (Rizzolo et al., 1985; Hobman et al., 1992). The nuclear rim staining suggests that the first transmembrane region of LBR is sufficient for sorting β-galactosidase to the inner nuclear membrane.

To investigate whether downstream transmembrane regions could substitute for the sorting determinant in the first transmembrane region we made an internally truncated LBR construct in which residues 206 to 279 were deleted. This construct (p58ΔTM1+2) was deliberately designed to lack transmembrane regions 1 and 2, rather than just transmembrane region 1, because it would be expected to yield a similar membrane orientation as full-length LBR. Immunolocalization showed a diffuse staining throughout the cytoplasm,

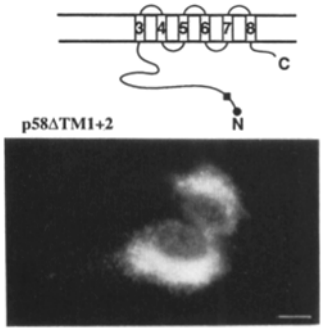

Figure 7. Transmembrane regions 3 to 8 do not contain a sorting signal. COS-1 cells transfected with p58ΔTM1+2 were fixed and permeabilized 40 h after transfection and processed for indirect immunofluorescence microscopy by staining with rabbit anti-p58 antibodies, followed by anti-rabbit IgG-FITC. Bar, 10 μm.

but no exclusive nuclear rim staining (Fig. 7). These data support the assignment of LBR's first transmembrane region as a determinant for sorting to the inner nuclear membrane and further demonstrate that the nucleoplasmic domain, per se, lacks determinants for sorting to the nuclear rim (see above).

Discussion

Our data here indicate that the first transmembrane region containing the first transmembrane segment plus flanking residues on either side contribute the determinants that sort LBR to the inner nuclear membrane. Surprisingly, the nucleoplasmic domain as well as the remaining seven transmembrane regions do not appear to contain sorting determinants, at least not under the experimental conditions used here, where the sorting of exogenously introduced LBR and LBR mutants is assessed in a background of endogenously expressed wild type LBR.

The mechanism by which LBR's first transmembrane region would specify sorting from its site of cotranslational insertion, namely the RER and the outer nuclear membrane, to the inner nuclear membrane remains to be investigated. It has been known for some time now (Furthmayr and Marchesi, 1976; Deisenhofer et al., 1985; Bormann et al., 1989; Lemmon et al., 1992) that a transmembrane α-helix is capable of specific interaction with another transmembrane α-helix. One possibility is that the transmembrane α-helix of a specific "sorting receptor" recognizes the transmembrane α-helix of LBR. Lateral movement of the complex along the outer nuclear membrane and the pore membrane domain of the nuclear envelope would carry LBR to the inner nuclear membrane where it might be dissociated from the "sorting receptor" and be retained through its interaction with lamin B and/or other intranuclear components. Another possibility is that the first transmembrane segment of one LBR molecule would specifically interact with the first transmembrane segment of another LBR molecule to form a homodimer and a binding site for lamin B and/or other intranuclear components. Homodimerization could occur immediately following integration into the RER/outer nuclear membrane followed by a random lateral diffusion of the homodimer along the outer nuclear membrane and the pore membrane domain of the nuclear envelope and ligand induced retention at the inner nuclear membrane. Alternatively, LBR monomers may reach the inner nuclear membrane, again via random lateral diffusion along the pore membrane domain of the nuclear envelope followed by a ligand (lamin B and/or intranuclear components) stabilized homodimerization and retention in the inner nuclear membrane. Similar scenarios can be considered if the first transmembrane segment of LBR would specifically recognize the transmembrane segment of another integral membrane protein to form a heterodimer.

The specific structural features of the first transmembrane helix region of LBR that mediate specific interaction with another transmembrane helix remain to be determined. The hydrophobic transmembrane segment is free of charged residues eliminating ion pair formation as a means for interhelical association. Interestingly, there is a cysteine (Cys 226) at the cisternal end of the transmembrane segment (see Fig. 6 *B*). Being exposed to an oxidizing environment this cysteine could potentially form a disulfide bond with the similarly located cysteine of the first transmembrane segment of a second LBR (or another protein). An example of this has been described recently, where disulfide linked dimerization of the ζ chain subunit of the T cell antigen receptor was shown to be mediated by a cysteine located at the lumenal end of the transmembrane domain (Rutledge et al., 1992). We are currently investigating the role of Cys 226 in LBR dimerization.

There are several precedents for a transmembrane segment serving as a sorting determinant. The first of three transmembrane segments of a viral protein has been shown to be the topogenic determinant for sorting to the *cis*-Golgi membrane (Machamer and Rose, 1987; Swift and Machamer, 1991). Moreover, the single transmembrane segments of two glycosyltransferases in the *trans*-Golgi have been identified as sorting determinants (Munro, 1991; Nilsson et al., 1991; Teasdale et al., 1992; Wong et al., 1992). And, the single transmembrane segment of gp210 serves as principal determinant for sorting to the pore membrane domain of the nuclear envelope (Wozniak and Blobel, 1992). In none of these cases has the precise mechanism by which the transmembrane segment functions in sorting been established.

Our finding here that the nucleoplasmic domain of LBR does not contain determinants for sorting to the inner nuclear membrane was unexpected. As mentioned in the introduction, the nucleoplasmic domain, by virtue of its topology, is an attractive candidate for containing LBR's lamin B binding site. However, the finding that the nucleoplasmic domain, although able to localize to the nucleoplasm, is not able to localize to the nuclear rim suggests that, per se, it is unable to compete with LBR for lamin B binding and thereby to attain the status of a "peripheral" membrane protein of the inner nuclear membrane. The ability of the nucleoplasmic domain to localize to the nucleoplasm remains to be investigated. Although this domain does not contain a minimal consensus nuclear localization signal (Chelsky et al., 1989), the protein is small enough so that it might diffuse into the nucleus (Paine et al., 1975) and localize there by retention. The distribution of the protein throughout the nucleoplasm suggests an affinity for a ubiquitous nuclear component, such as chromatin rather than a peripheral one like the lamina. Affinity for chromatin is consistent with the SPXX motifs that are present in the nucleoplasmic domain (Worman et al., 1990) and that are common to many DNA binding proteins.

Our findings here that the transmembrane region of LBR specifies localization to the inner nuclear membrane adds another example to the growing list of transmembrane segments that carry sequence-specific information rather than just serving as nonspecific tethering devices. The specific in-

formation residing in the primary structure of the transmembrane α-helix appears to be decoded via specific interaction with another transmembrane α-helix to form dimers. Such dimerization has been shown to be important in signal transduction (reviewed by Ullrich and Schlessinger, 1990; De Vos et al., 1992) and is likely to be involved in protein degradation (Bonifacino et al., 1990*a*,*b*) and in protein topogenesis (see above).

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