The Amino-Terminal Domain of the Lamin B Receptor Is a Nuclear Envelope Targeting Signal

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Abstract. The lamin B receptor (LBR) is a polytopic protein of the inner nuclear membrane. It is synthesized without a cleavable amino-terminal signal sequence and composed of a nucleoplasmic amino-terminal domain of 204 amino acids followed by a hydrophobic domain with eight putative transmembrane segments. To identify a nuclear envelope targeting signal, we have examined the cellular localization by immunofluorescence microscopy of chicken LBR, its amino-terminal domain and chimeric proteins transiently expressed in transfected COS-7. Full-length LBR was targeted to the nuclear envelope. The amino-terminal domain, without any transmembrane segments, was transported to the nucleus but excluded from the nucleolus. When the amino-terminal domain of LBR was fused to the amino-terminal side of a transmembrane segment of a type II integral membrane protein of the ER/plasma membrane, the chimeric protein was targeted to the nuclear envelope, likely the inner nuclear membrane. When the amino-terminal domain was deleted from LBR and replaced by α-globin, the chimeric protein was retained in the ER. These findings demonstrate that the amino-terminal domain of LBR is targeted to the nucleus after synthesis in the cytoplasm and that this polypeptide can function as a nuclear envelope targeting signal when located at the amino terminus of a type II integral membrane protein synthesized on the ER.

THE topology of integral membrane proteins synthesized on the ER is established by signal and stop transfer sequences that determine which domains traverse aqueous pores in the ER membrane and which domains remain localized in the membrane (Blobel, 1980; Simon and Blobel, 1991). Some of these proteins contain specific sequences that confer retention in the ER (Paabo et al., 1987; Nilsson et al., 1989; Jackson et al., 1990). Others are targeted to the *cis*-Golgi network by ER-derived membrane vesicles, and some of these integral membrane proteins contain sequences that confer retention in the Golgi membranes (Machamer and Rose, 1987; Nilsson et al., 1991; Wong et al., 1992). Most integral membrane proteins that reach the Golgi complex are sorted into different populations of membrane vesicles that emerge from the TGN to become secretory storage vesicles or to fuse with lysosomes or the plasma membrane (Griffiths and Simons, 1986; Klausner, 1989; Kornfeld and Mellman, 1989; Rothman and Orci, 1992). In epithelial cells, additional sorting mechanisms are operative that target protein-containing membrane vesicles to either the apical or basolateral membranes (Rodriguez-Boulan and Nelson, 1989).

Some integral membrane proteins synthesized on the ER are targeted to the various membrane domains of the nuclear envelope. The nuclear envelope is composed of three distinct membrane domains: the inner nuclear membrane, the outer nuclear membrane and the nuclear pore membranes (Franke et al., 1981; Gerace and Blobel, 1982). These three membrane domains are interconnected, but unique integral membrane proteins are found in each that are not found in the others (Gerace et al., 1982; Worman et al., 1988, 1990; Senior and Gerace, 1988; Harel et al., 1989; Courvalin et al., 1990). Proteins of the outer nuclear membrane are shared with the continuous ER membrane (Amar-Costesec et al., 1974) and outer nuclear membrane proteins have not been identified that are not also found in the ER. As the ER, outer, pore, and inner nuclear membrane domains are continuous, integral membrane proteins synthesized on the ER can reach all of the nuclear envelope membrane domains by diffusion in the proteolipid bilayer. The signals that target integral membrane proteins to their specific nuclear envelope membrane domains are not known.

The lamin B receptor (LBR)[1] is a polytopic protein of the inner nuclear membrane with a basic nucleoplasmic amino-terminal domain and a hydrophobic domain that contains eight putative transmembrane segments (Worman et al., 1990). LBR is synthesized without a cleavable amino-terminal signal sequence, and like type II integral membrane proteins of the ER/plasma membrane that contain uncleaved internal signal sequences, its amino-terminal domain faces the contralumenal or nucleo-cytoplasmic side of the mem-

1. *Abbreviations used in this paper*: LBR, lamin B receptor; NLS, nuclear localization signal.

brane (Worman et al., 1990). To identify a nuclear envelope targeting signal of LBR, we have transfected cells with plasmids that transiently express different domains and chimeric constructs of this protein.

Materials and Methods

Plasmid Construction

Expression constructs were made in plasmid pSVK3 (Pharmacia Fine Chemicals, Piscataway, NJ) which contains a multiple cloning site downstream from the SV-40 early promoter. Some cDNA sequences were generated by the PCR performed as described (Saiki et al., 1987) using the GeneAmp Kit (Perkin-Elmer Corp., Norwalk, CT). Parameters for PCR were denaturation at 94°C for 1 min, annealing at 50°C for 2 min and extension at 72°C for 3 min for 25 cycles. Unless otherwise indicated, standard methods (Sambrook et al., 1989) were used for DNA purification, restriction endonuclease digestion, ligation, transformation, and preparation of plasmid DNA.

The cDNA sequence of chicken LBR and descriptions of LBR clones have been previously published (Worman et al., 1990). To construct plasmid LMBR that codes for full-length chicken LBR, a PCR product from nucleotide +1210 to nucleotide +1914 was amplified using as template a mixture of DNA from previously described clones M-3 and M-7. A HindIII site was engineered at the 5′ end of the antisense primer. The product was purified from the reaction mixture using the GeneClean II Kit (Bio 101, Inc., Vista, CA), cut with NheI (NheI site at nucleotide +1303 of LBR cDNA) and HindIII, purified again and ligated into LBR clone DJ-5 that was cut with NheI and HindIII. The resulting recombinant clone, which contained cDNA coding for full-length LBR, was digested with EcoRI and SalI and the cDNA insert isolated from agarose gels and ligated into the EcoRI/SalI site of pSVK3.

Plasmid AT was constructed to express the amino-terminal domain of chicken LBR. A PCR product from nucleotides −42 to +625 of LBR cDNA was amplified using clone DJ-5 as template. The sense primer had an EcoRI site at the 5′ end and the antisense primer a complementary stop codon preceded by a SalI site at its 5′ end. The amplified product was purified from the reaction mixture using GeneClean, digested with EcoRI and SalI, purified again and ligated into the EcoRI/SalI site of pSVK3.

A previously described cDNA clone for chicken hepatic lectin in plasmid pSP64 (Mellow et al., 1988) was used to construct plasmid CHL. This clone was a gift from Dr. K. Drickamer (Columbia University College of Physicians and Surgeons, New York). The cDNA insert was excised from the plasmid by restriction digestion with EcoRI, isolated by agarose gel electrophoresis and ligated into the EcoRI site of pSVK3.

Plasmid LMBR-CHL was constructed to express the first 191 amino acids of the chicken LBR followed by the transmembrane domain and portion of the carboxy-terminal domain of chicken hepatic lectin. DNA from nucleotide 183 to nucleotide 510 of chicken hepatic lectin cDNA that coded for amino acids 24 to 131 (numbering according to Mellow et al., 1988) was amplified by PCR using the pSP64 clone as template. The sense primer used in this reaction had a BspEI engineered into its 5′ end and the antisense primer a complementary stop codon preceded by an ApaI site at its 5′ end. The product was purified from the reaction mixture by phenol:chloroform extraction, digested with BspEI and ApaI, purified again and ligated into plasmid LMBR that was digested with BspEI and ApaI (LBR cDNA has a BspEI restriction site at nucleotide +567).

Complementary DNA coding for chimpanzee α-globin was cloned into pSVK3 to generate plasmid AG. A plasmid containing a cDNA insert coding for chimpanzee α-globin (Rothman et al., 1988) was a gift from Dr. V. R. Lingappa (University of California, San Francisco). This plasmid was used as template for PCR to generate α-globin cDNA from the initiator ATG through the TAA stop codon (cDNA sequence according to Liebhaber and Begley, 1984). The sense primer had an EcoRI site engineered into its 5′ end and the antisense primer a SalI site at its 5′ end. The product was purified from the reaction mixture by phenol:chloroform extraction, digested with EcoRI and SalI, purified again and ligated into the EcoRI/SalI site of pSVK3.

Plasmid AG-LMBR was constructed to express a chimeric protein in which the first 191 amino acids of LBR were replaced by chimpanzee α-globin. α-globin cDNA was amplified by PCR as described in the preceding paragraph but with a different antisense primer that did not extend to the TAA stop codon and had a BspEI site added in frame 5′ to the last three nucleotides of the coding sequence. The PCR product was purified from the reaction mixture by phenol:chloroform extraction, digested with EcoRI and BspEI, purified again and ligated into EcoRI/BspEI digested plasmid LMBR.

All recombinant plasmids were analyzed by restriction analysis. Plasmids in which PCR was used to generate coding sequences were also analyzed by DNA sequencing performed on a Model 373A Automated Sequencer (Applied Biosystems, Inc., Foster City, CA) at The Mount Sinai School of Medicine DNA Core Facility (New York).

Cell Culture and Transfection

COS-7 cells were grown in DME media containing 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mM l-glutamine. For transfection, cells were grown to 60% confluency on 60-mm-diameter petri dishes. Cells were transfected by the calcium precipitation method as described (Sambrook et al., 1989). The precipitated plasmid DNA (10 μg) was left on each petri dish for 5 h. Cells were then washed with PBS, split into 35-mm-diameter petri dishes and maintained in culture for 12 to 72 h before fixation for immunofluorescence microscopy.

Antibodies

Rabbit antibodies against human hemoglobin that recognize chimpanzee α-globin were obtained from Dako Corporation (Carpinteria, CA). Rabbit antibodies against chicken hepatic lectin were a gift from Dr. K. Drickamer and have been previously described (Loeb and Drickamer, 1987). Antibodies that recognize the amino-terminal domain of chicken LBR were made against a synthetic polypeptide of sequence S-P-K-Q-R-K-S-Q-S-S-S-S-S (The Rockefeller University/Howard Hughes Medical Institute Biopolymer Facility, New York, NY). The polypeptide was conjugated to keyhole limpet hemocyanin (Calbiochem-Novabiochem Corp., La Jolla, CA) by previously described methods (Muller et al., 1982). The conjugate was injected into rabbits to produce polyclonal antibodies as described (Posnett et al., 1988). Anti-LBR antibodies were purified from rabbit serum by affinity chromatography against the synthetic polypeptide coupled to Affi-Gel (Bio Rad Labs, Hercules, CA). Coupling using EDAC reagent was performed according to the manufacturer's instructions. Affinity chromatography was performed as described elsewhere (Harlow and Lane, 1988). Anti-LBR antibodies were characterized by immunoblotting of proteins of chicken erythrocyte nuclear envelopes, rat liver nuclear envelopes and COS-7 cell lysates. Chicken erythrocyte nuclear envelopes were prepared as described for turkey erythrocytes (Worman et al., 1988) and rat liver nuclear envelopes were also prepared by methods used previously (Courvalin et al., 1990). COS-7 cell lysates were made by scraping cells off petri dishes and immediately boiling them in SDS-PAGE sample buffer (Laemmli, 1970). SDS-PAGE was performed according to Laemmli (1970). References for immunoblotting can be found elsewhere (Courvalin et al., 1990).

Immunofluorescence Microscopy

Transfected cells were grown to 60% confluency on 35-mm-diameter petri dishes. Cells were washed three times with PBS, fixed with 3% formaldehyde/PBS for 30 min and washed three times with PBS/0.1% Triton X-100 (solution A). Cells were then incubated with PBS/0.1% Triton X-100/2% BSA (solution B) for 60 min and then for 90 min with the appropriate antibody at a 1:100 dilution in solution B. Cells were then washed five times with solution A, incubated for 60 min with a 1:100 dilution of fluorescein-labeled donkey anti-rabbit IgG (Amersham, Arlington Heights, IL) in solution B plus 2 μg/ml 4,6-diamidino-2-phenylindole (DAPI), and again washed five times with solution A. Coverslips were mounted with a 2% wt/vol solution of 1,4-diazabicyclo-(2.2.2)-octane in 85% glycerol/15% 8 mM Tris-HCl (pH 8.6). Immunofluorescence microscopy was performed on a Zeiss Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY). T-MAX 3200 or T-MAX 400 film (Eastman Kodak Co., Rochester, NY) were used for photography.

Epitope Accessibility in Digitonin-permeabilized Cells

To remove the plasma membrane but keep the nuclear membranes intact, cells were permeabilized with digitonin as described by Adam et al. (1990). A single petri dish of COS-7 cells was transfected with the desired plasmid, split into two dishes after 5 h and then grown as described above. After 24 h in culture, one dish was prepared for immunofluorescence microscopy as usual. The other dish of similarly transfected cells was washed three times with PBS and the cells were then permeabilized for 5 min with 40 μg/ml

of digitonin (Calbiochem-Novabiochem Corp., La Jolla, CA) in PBS at 4°C (digitonin was diluted into PBS from a 20 mg/ml stock in DMSO). After permeabilization, cells were washed again with PBS and prepared for immunofluorescence microscopy as usual except that the cells were not fixed and Triton X-100 was not present in solutions A and B. Immunofluorescence microscopy was performed as above.

Materials

Unless otherwise indicated, routine chemical reagents were obtained from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Restriction enzymes, restriction enzyme buffers and DNA ligase were obtained from New England Biolabs (Beverly, MA). FBS was obtained from JRH Biosciences (Lenexa, KS). Other media and reagents for cell culture were obtained from either GIBCO BRL (Gaithersburg, MD) or Mediatech (Washington, DC).

Results

Anti-LBR Antibody Characterization

Because COS-7 cells contain endogenous LBR, antibodies were needed for transfection experiments that recognize chicken but not mammalian LBR. For this purpose, antibodies were raised against a synthetic polypeptide corresponding to amino acids 59–71 of chicken LBR. Proteins of COS-7 cell lysates and chicken erythrocyte nuclear envelopes were separated by SDS-PAGE (Fig. 1 *a*) and on immunoblots of these separated proteins, the anti-polypeptide antibodies recognized chicken LBR but did not recognize any proteins in COS-7 cell lysates (Fig. 1 *b*). These antibodies also did not recognize proteins in nuclear envelope preparations from rat liver (data not shown) and did not give nuclear rim fluorescence in immunofluorescence microscopy of cultures of untransfected COS-7 cells (data not shown) or in numerous cells in transfection experiments that did not likely take up plasmid DNA (for example see Fig. 5). Since these antibodies did not apparently recognize mammalian LBR or stain the nuclear envelopes of COS-7 cells, they were suitable to follow the expression of the chicken protein in transfection experiments.

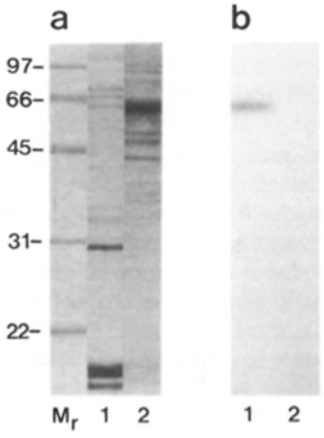

Figure 1. Anti-polypeptide antibodies recognize chicken but not mammalian LBR. (*a*) Coomassie blue-stained SDS-polyacrylamide slab gel (10.5% acrylamide) showing molecular mass standards (M_r), proteins of chicken erythrocyte nuclear envelopes (lane *1*) and proteins of COS-7 cell lysates (lane 2). Molecular mass standards are indicated in kD. (*b*) Autoradiogram of immunoblot of preparations identical to those shown in panel *a* using anti-LBR antibodies against proteins of chicken erythrocyte nuclear envelopes (lane *1*) and proteins of COS-7 cell lysates (lane 2). The antibodies recognize LBR in chicken erythrocyte nuclear envelopes but do not recognize any proteins in COS-7 cell lysates.

Protein Targeting in Transfected COS-7 Cells

Constructs in plasmid pSVK3 were designed to transiently express in COS-7 cells the proteins shown in Fig. 2. LMBR expressed full-length chicken LBR, a polytopic protein of the inner nuclear membrane that is synthesized without a cleavable amino-terminal signal sequence and has a nucleoplasmic amino-terminal domain followed by a hydrophobic domain with eight putative transmembrane segments that can likely function as uncleaved internal signal sequences (Worman et al., 1990). AG expressed chimpanzee α-globin, a 142-amino acid polypeptide that is a subunit of the cytoplasmic protein hemoglobin (Liebhaber and Begley, 1984). CHL expressed chicken hepatic lectin, a type II integral membrane protein of the ER/plasma membrane that is synthesized without a cleavable signal sequence and has an amino terminus that faces the cytoplasm, one transmembrane segment that likely functions as an uncleaved internal signal sequence and carboxy terminus that faces the ER lumen/extracellular milieu (Chiacchia and Drickamer, 1984; Mellow et al., 1988). AT expressed the amino-terminal domain of LBR from the initiation methionine to the arginine that immediately precedes the first putative transmembrane segment. CHL-LMBR expressed a chimeric protein that contained the first 191 amino acids of LBR followed by the transmembrane domain and a portion of the carboxy-terminal domain (up to amino acid 131) of chicken hepatic lectin. AG-LMBR expressed a chimeric protein in which all 142 amino acids of chimpanzee α-globin replaced the first 191 amino-terminal amino acids of LBR.

To determine the immunofluorescence patterns for full-length LBR, chicken hepatic lectin and α-globin, three proteins of known cellular localization, COS-7 cells were transfected with plasmids LMBR, CHL, and AG. LBR was detected only in the nuclear envelope of transfected cells as demonstrated by rim fluorescence of the nucleus (Fig. 3 *a*). A rim-staining fluorescence pattern is characteristic of extrinsic and integral proteins of the inner nuclear membrane (Gerace et al., 1978; Worman et al., 1988; Senior and Gerace, 1988; Courvalin et al., 1990). α-globin was detected in the cytoplasm of cells transfected with plasmid AG (Fig. 3 *b*). The fluorescence pattern of discrete dots throughout the

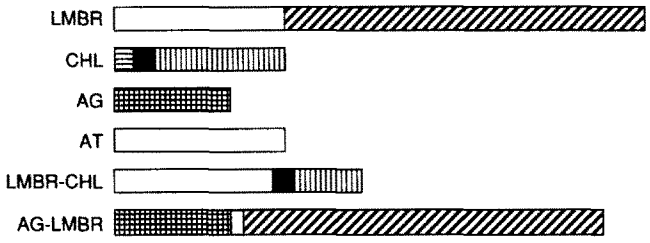

Figure 2. Schematic diagrams of proteins expressed by the plasmid constructs used in transfection experiments. Domains of the proteins are represented by: white, nucleoplasmic amino-terminal domain of LBR; diagonal stripes, hydrophobic domain of LBR with eight putative transmembrane segments; horizontal stripes, cytoplasmic amino-terminal domain of chicken hepatic lectin; black, transmembrane segment of chicken hepatic lectin; vertical stripes, carboxy-terminal domain of chicken hepatic lectin; checkered, α-globin. The amino-terminal domain of each protein is at the left.

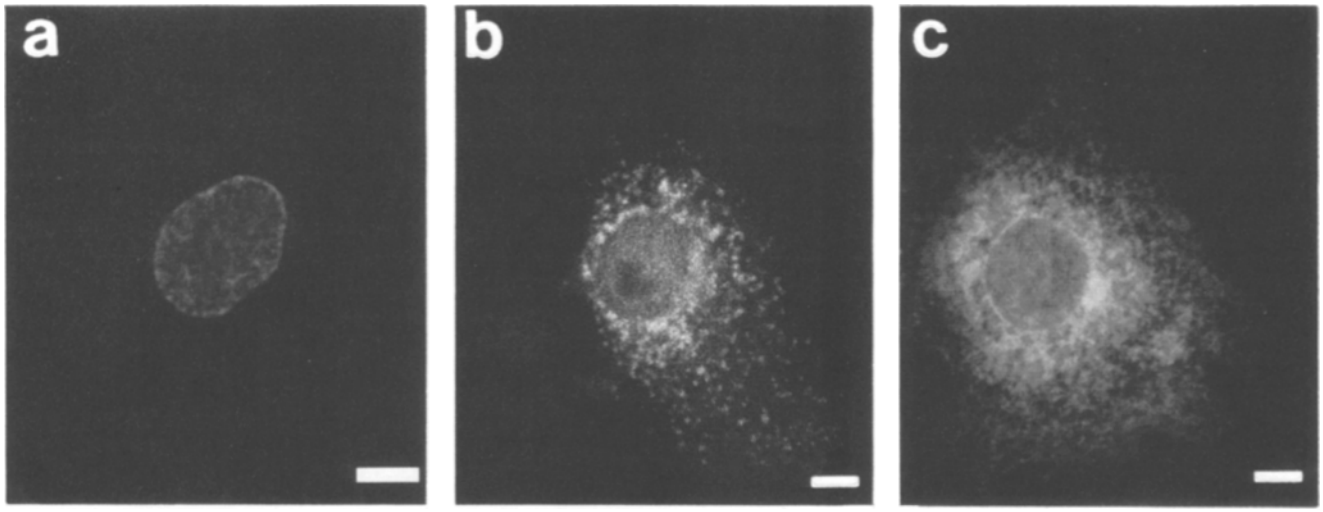

Figure 3. Cellular localization of LBR, α-globin and chicken hepatic lectin. Shown are immunofluorescence photomicrographs of COS-7 cells transfected with plasmids LMBR (*a*), AG (*b*), and CHL (*c*) that express LBR, α-globin and chicken hepatic lectin respectively. Anti-LBR antibodies were used in *a*, anti-α-globin antibodies in *b* and anti-chicken hepatic lectin antibodies in *c*. Some weak background nuclear fluorescence is seen with the anti-α-globin and anti-chicken hepatic lectin antibodies in transfected and nontransfected cells. Bars, 4 μm.

cytoplasm may result from precipitation or aggregation of the expressed polypeptide. Cells expressing chicken hepatic lectin demonstrated fluorescent labeling of the ER and nuclear envelope (Fig. 3 *c*), a pattern that has been previously reported for its expression in transfected fibroblasts (Mellow et al., 1988) and is expected for a protein of the ER and outer nuclear membrane.

COS-7 cells were transfected with plasmid AT to determine if the nucleoplasmic amino-terminal domain of LBR lacking transmembrane segments would be transported to the nucleus. This polypeptide was detected only in the nucleus of transfected cells (Fig. 4). A diffuse fluorescence labeling of the entire nucleus, similar to that seen with the DNA-binding dye DAPI, was observed. The fluorescence intensity of the nucleolus was relatively decreased indicating that the polypeptide is excluded from this organelle. After transfection and growth in culture for up to 72 h, only nuclear fluorescence was observed without significant cytoplasmic labeling or disruption of nuclear architecture. These experiments demonstrate that the LBR amino-terminal domain is transported to the nucleus after synthesis in the cytoplasm.

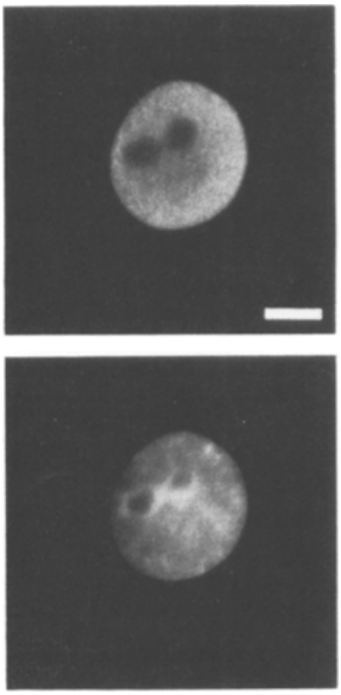

Figure 4. The amino-terminal domain of LBR is transported to the nucleus. Top panel shows immunofluorescence photomicrograph using anti-LBR antibodies of COS-7 cells transfected with plasmid AT that expressed the amino-terminal domain of LBR. Bottom panel shows DAPI fluorescence for the same cell. Bar, 4 μm.

To determine if the amino-terminal domain of LBR can function as a nuclear envelope targeting signal for an integral membrane protein, it was attached to the transmembrane segment of a type II protein of the ER/plasma membrane. COS-7 cells were transfected with plasmid LMBR-CHL that codes for a chimeric protein containing the amino-terminal domain of LBR fused to the amino-terminal side of the transmembrane segment of chicken hepatic lectin. In cells transfected with this plasmid, nuclear rim fluorescence without ER labeling was observed consistent with an inner nuclear membrane localization (Fig. 5 *a*). No appreciable ER fluorescence was observed even 72 h after transfection. Hence, the amino-terminal domain of LBR can function as a nuclear envelope targeting signal and direct an integral membrane protein synthesized on the ER to the inner nuclear membrane. To confirm this hypothesis, the majority of the amino-terminal domain was deleted from full-length LBR and replaced by α-globin. The expressed chimeric protein was detected in the ER and nuclear envelope of transfected cells (Fig. 5 *b*). An exclusive fluorescence labeling of the nuclear envelope, which was seen with LBR and the LBR-chicken hepatic lectin chimeric protein, was never observed even when cells were examined only 12 h after transfection. Fluorescence labeling of the ER and nuclear envelope does not exclude the possibility that some of the expressed protein is in the inner nuclear membrane or nuclear pore membranes; however, a large portion of the α-globin–LBR chimeric protein is retained in the ER. Therefore, even if some of the ex-

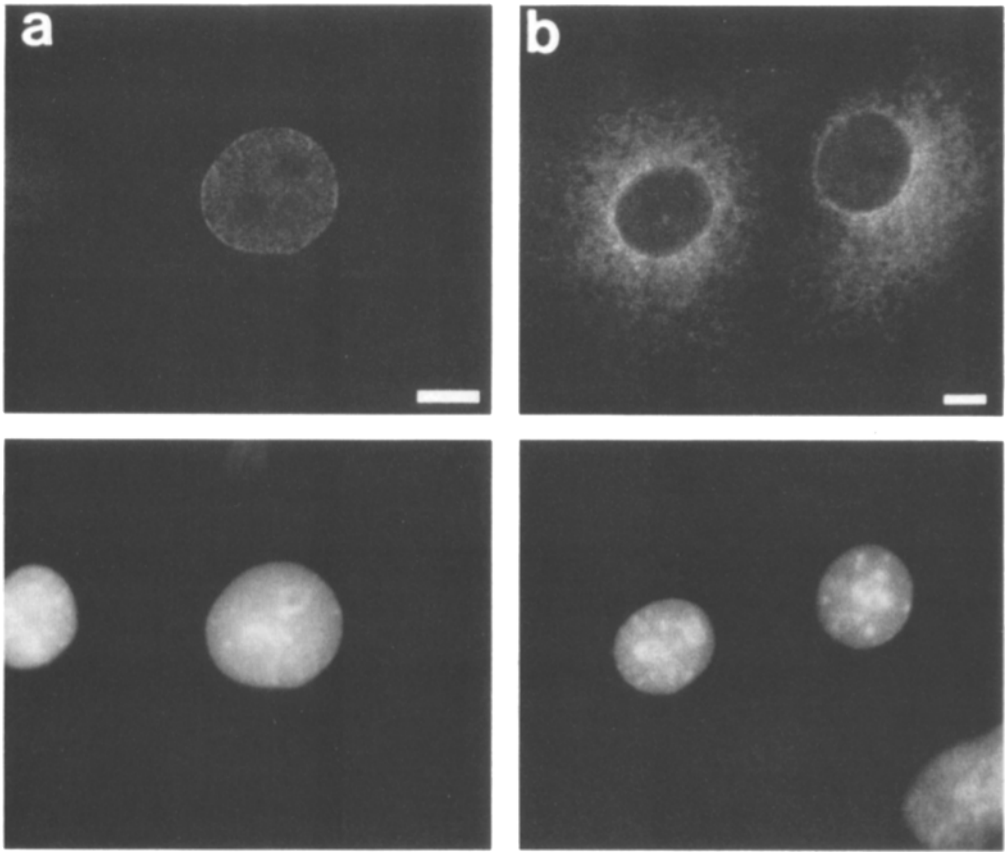

Figure 5. The amino-terminal domain of LBR is a nuclear envelope targeting signal for integral membrane proteins. Top panels show immunofluorescence photomicrographs of COS-7 cells transfected with plasmids LMBR-CHL (*a*) and AG-LMBR (*b*) that express chimeric proteins with and without the LBR amino-terminal domain (see text and Fig. 2). Antibodies against LBR were used in *a* and antibodies against α-globin in *b*. Bottom panels show DAPI fluorescence for the same microscopic fields as shown above. In *a* the nucleus of an untransfected cell that is not recognized by anti-LBR antibodies can be identified by DAPI fluorescence. Bars, 4 μm.

pressed chimeric protein reaches the inner nuclear membrane, its targeting signal is weaker than that of the two proteins that contain the amino-terminal domain of LBR.

Epitope Accessibility Experiments Suggest That LBR and LBR-Chicken Hepatic Lectin Chimeric Proteins Are Localized to the Inner Nuclear Membrane

The rim-staining fluorescence patterns seen for transfected cells expressing LBR and the LBR–chicken hepatic lectin chimeric protein are consistent with a location in the inner nuclear membrane, but could also be seen if these proteins were localized to the nuclear pore membranes or to the outer nuclear membrane. To further confirm an inner nuclear membrane localization of these polypeptides, their accessibility to antibodies was determined in unfixed digitonin-permeabilized cells. Treatment of cultured cells with appropriate amounts of digitonin removes the plasma membrane but leaves the nuclear envelope intact (Adam et al., 1990). In unfixed, digitonin-permeabilized cells, protein antigens

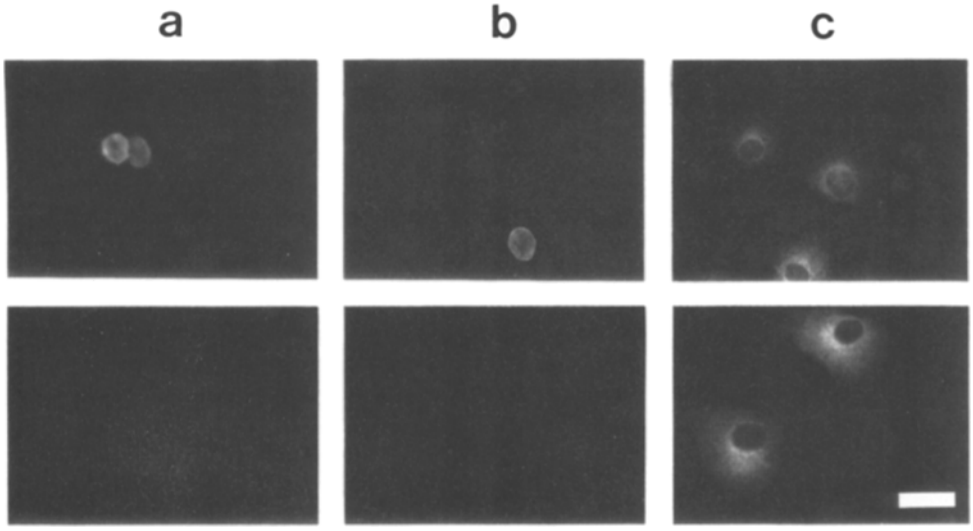

Figure 6. Accessibility of LBR, LBR–chicken hepatic lectin, and α-globin-LBR to antibodies in digitonin-permeabilized cells. COS-7 cells transfected with LMBR (*a*), LMBR-CHL (*b*), and AG-LMBR (*c*) are shown. Upper panels show representative immunofluorescence micrographs of fixed cells permeabilized with Triton X-100. Bottom panels show representative immunofluorescence micrographs for similarly transfected cells that were permeabilized with digitonin, not fixed and not exposed to Triton X-100. Antibodies against LBR were used in *a* and *b* and antibodies against α-globin in *c*. Bar, 20 μm.

located in the nuclear pore complexes and cytoplasm are recognized by antibodies in immunofluorescence microscopy, whereas antigens located on the inner side of the nuclear envelope are not accessible to the antibodies (Adam et al., 1990).

COS-7 cells were transfected with plasmids LMBR, LMBR-CHL, and AG-LMBR, respectively, and the transfected cells were split into two petri dishes. Cells in one petri dish of each pair were prepared for immunofluorescence microscopy in standard fashion with fixation and exposure to Triton X-100 that permits antibodies access to intranuclear antigens. The other half of the cells were not fixed, were permeabilized with digitonin and prepared for immunofluorescence microscopy in the absence of Triton X-100. For cells transfected with LMBR (Fig. 6 *a*) and LMBR-CHL (Fig. 6 *b*) that were fixed and treated with Triton X-100 (*upper panels*), rim fluorescence was observed in transfected cells. In unfixed, digitonin-permeabilized cells that were not exposed to Triton X-100 (*lower panels*), fluorescence was not observed in any cells. In contrast, cells transfected with AG-LMBR (Fig. 6 *c*) showed staining in unfixed, digitonin-permeabilized cells (*lower panel*) as well as cells fixed and exposed to Triton X-100 (*upper panel*). These data suggest that in the digitonin-permeabilized cells, the α-globin-LBR chimeric protein is accessible to antibodies that can pass through the disrupted plasma membrane. LBR and LBR-chicken hepatic lectin are apparently not accessible to antibodies in digitonin-permeabilized cells, consistent with a localization of these polypeptides on the inside of the nuclear envelope in the inner nuclear membrane.

Discussion

The findings in the present study demonstrate that the nucleoplasmic amino-terminal domain of the inner nuclear membrane LBR is transported to the nucleus after synthesis in the cytoplasm. This polypeptide is sufficient to target a type II integral membrane protein to the nuclear envelope inner membrane domain. Deletion of the amino-terminal domain from LBR weakens and perhaps abolishes the ability of the protein to reach the inner nuclear membrane.

The present findings must be interpreted in light of the putative topology of LBR. LBR is synthesized without a cleavable amino-terminal signal sequence (Worman et al., 1990) and therefore the amino-terminal domain likely faces the cytoplasm when synthesized on the ER membrane. With this topology, the first putative transmembrane domain would function as both an uncleaved signal sequence and a stop transfer sequence (Blobel, 1980). Hence, LBR is synthesized on the ER membrane with a topology analogous to type II integral membrane proteins of the plasma membrane. This topology of LBR is supported by the finding that a residue in its amino-terminal domain is phosphorylated in vivo by $p34^{cdc2}$ protein kinase (Courvalin et al., 1992), an enzyme that is present in the nucleus and cytoplasm and not likely the ER lumen or the perinuclear space. When reaching the inner nuclear membrane, the amino-terminal domain of LBR would have a nucleoplasmic orientation like the amino termini of type II plasma membrane proteins have a cytoplasmic orientation. As LBR amino-terminal domain can reach the nucleus after synthesis in the cytoplasm, cytoplasmically exposed amino-terminal domain should also be able to reach the nucleus by the same mechanism when attached to transmembrane segments in the ER membrane. The nuclear translocation of LBR amino-terminal domain would then "drag" the transmembrane segments of the protein that remain embedded in the proteolipid bilayer to the inner nuclear membrane. The ultrastructure of the nuclear pore complex would have to be such that there is no steric hindrance and that membrane-embedded proteins can freely diffuse in the pore membrane domain.

How does the amino-terminal domain of LBR reach the nucleus after synthesis in the cytoplasm? Many nuclear proteins contain short stretches of basic amino acids called nuclear localization signals (NLS's) that are responsible for nuclear transport (Garcia-Bustos et al., 1991). NLS's can direct transport to the nucleus when fused to normally cytoplasmic proteins (Goldfarb et al., 1986). Most NLS's are similar to the sequence P-K-K-K-R-K-V found in SV-40 large T antigen (Kalderon et al., 1984). Cytosolic and pore complex proteins that bind to these NLS's target proteins to the nuclear pores and facilitate transport into the nucleus (Silver, 1991; Adam and Gerace, 1991; Moore and Blobel, 1992). Proteins that contain SV40-type NLS's transported to the nucleus by this mechanism may first be concentrated in the nucleolus after passing through the pores (Breeuwer and Goldfarb, 1990; Meier and Blobel, 1990; Moore and Blobel, 1992) and recent evidence has shown that an NLS binding protein may shuttle between the nucleolus and the cytoplasm (Meier and Blobel, 1992). The LBR amino-terminal domain does not contain a stretch of amino acids similar to the "classical" NLS of SV-40 large T antigen, and the finding that the polypeptide is excluded from the nucleolus also suggests that its nuclear transport does not involve proteins that shuttle between the cytoplasm and nucleolus. It would also be unlikely that translocation to the nucleolus could be achieved for intact LBR where most of the protein would be embedded in the nuclear envelope membranes.

In addition to the "classical" NLS found in SV-40 large T antigen, up to 40% of nuclear proteins use a second class of NLS exemplified by the bipartite NLS of nucleoplasmin (Robbins et al., 1991). Two sequences in the amino-terminal domain of LBR (from Arg^{63} to Arg^{79} and from Arg^{93} to Lys^{108}) roughly fit the sequence of the nucleoplasmin-like bipartite motif that can function as an NLS (Robbins et al., 1991). Therefore, the amino-terminal domain of LBR may use a nucleoplasmin-like bipartite NLS to enter the nucleus. Proteins that contain this type of NLS may use different cytoplasmic and nuclear pore complex receptor proteins and may not concentrate in the nucleolus during nuclear accumulation.

It is also possible that the LBR amino-terminal domain can accumulate in the nucleus by diffusion and ligand binding. As fluorescent-labeled dextrans with molecular masses <60 kD can passively diffuse through the nuclear pore complexes (Paine et al., 1975), the LBR amino-terminal domain is small enough ($\sim$22.5 kD) to enter the nucleus by diffusion. If LBR amino-terminal polypeptide can diffuse through the nuclear pores, its concentration in the nucleus may result from its ability to bind to nuclear components. In vitro experiments have shown that LBR binds to lamin B (Worman et al., 1988), and it is possible that the lamin B binding domain of the protein is in the nucleoplasmic amino terminus. If the free amino-terminal domain were binding only to

lamin B in vivo, a nuclear rim fluorescence pattern would be expected on immunofluorescence microscopy. The diffuse localization in the nucleus observed when free amino-terminal domain is expressed in transfected cells argues against lamin B binding being solely responsible for nuclear accumulation of this polypeptide. Therefore, in the nucleus in vivo, the LBR amino-terminal polypeptide may bind to other nuclear components in addition to lamin B. The amino-terminal domain of LBR also possesses characteristics of a chromatin binding protein (Worman et al., 1990) including a basic pI and three S/T-P-X-X motifs that have the capacity to bind to DNA (Suzuki et al., 1989). The diffuse staining of the nucleus in immunofluorescence microscopy suggests that the LBR amino-terminal domain may bind to chromatin and that this binding, in addition to lamin B binding, may be responsible for its concentration in the nucleus. When embedded in the ER membrane via a transmembrane segment after synthesis, the cytoplasmically exposed amino-terminal domain of LBR may cause the protein to diffuse through the interconnected proteolipid bilayers of the ER, outer nuclear and nuclear pore membranes to reach the inner nuclear membrane where the exposed amino terminus can bind to intranuclear components such as chromatin or lamin B.

In summary, the amino-terminal domain of the inner nuclear membrane LBR can function as a nuclear envelope targeting signal and can direct a type II integral membrane protein synthesized on the ER to the nuclear envelope, likely the inner nuclear membrane. The targeting polypeptide may be accumulated in the nucleus either by active transport used by proteins that contain nucleoplasmin-like bipartite NLS's or by diffusion and ligand binding. Future experiments examining the details of lamin B binding, chromatin binding, and in vitro transport of the LBR amino-terminal domain will help further elucidate the mechanism of how this polypeptide functions as a nuclear envelope targeting signal.

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References

Adam, S. A., and L. Gerace. 1991. Cytosolic proteins that specifically bind nuclear localization signals are receptors for nuclear import. *Cell.* 66:837–847.

Adam, S. A., R. S. Marr, and L. Gerace. 1990. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol.* 111:807–816.

Amar-Costesec, A., H. Beaufay, M. Wibo, D. Thinès-Sempoux, E. Feytmans, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. II. Preparation and composition of the microsomal fraction. *J. Cell Biol.* 61:201–212.

Blobel, G. 1980. Intracellular protein topogenesis. *Proc. Natl. Acad. Sci. USA.* 77:1496–1500.

Breeuwer, M., and D. S. Goldfarb. 1990. Facilitated nuclear transport of histone H1 and other small nucleophilic proteins. *Cell.* 60:999–1008.

Chiacchia, K. B., and K. Drickamer. 1984. Direct evidence for the transmembrane orientation of the hepatic glycoprotein receptors. *J. Biol. Chem.* 259:15440–15446.

Courvalin, J.-C., K. Lassoued, H. J. Worman, and G. Blobel. 1990. Identification and characterization of autoantibodies against the nuclear envelope lamin B receptor from patients with primary biliary cirrhosis. *J. Exp. Med.* 172:961–967.

Courvalin, J.-C., N. Segil, G. Blobel, and H. J. Worman. 1992. The lamin B receptor of the inner nuclear membrane undergoes mitosis-specific phosphorylation and is a substrate for $p34^{cdc2}$-type protein kinase. *J. Biol. Chem.* 267:19035–19038.

Franke, W. W., U. Scheer, G. Krohne, and E. Jarasch. 1981. The nuclear envelope and the architecture of the nuclear periphery. *J. Cell Biol.* 91:39s–50s.

Garcia-Bustos, J., J. Heitman, and M. N. Hall. 1991. Nuclear protein localization. *Biochim. Biophys. Acta.* 1071:83–101.

Gerace, L., and G. Blobel. 1982. Nuclear lamina and the structural organization of the nuclear envelope. *Cold Spring Harbor Symp. Quant. Biol.* 46:967–978.

Gerace, L., A. Blum, and G. Blobel. 1978. Immunocytochemical localization of the major polypeptides of the nuclear pore complex-lamina fraction. *J. Cell Biol.* 79:546–566.

Gerace, L., Y. Ottaviano, and C. Kondor-Koch. 1982. Identification of a major polypeptide of the nuclear pore complex. *J. Cell Biol.* 95:826–837.

Goldfarb, D. S., J. Gariepy, G. Schoolnik, and R. D. Kornberg. 1986. Synthetic peptides as nuclear localization signals. *Nature (Lond.).* 322:641–644.

Griffiths, G., and K. Simons. 1986. The trans Golgi network: sorting at the exit site of the Golgi complex. *Science (Wash. DC).* 234:438–443.

Harel, A., I. Zlotkin, S. Nainudel-Epszteyn, N. Feinstein, P. A. Fisher, and Y. Gruenbaum. 1989. Persistence of a major nuclear envelope antigen in an envelope-like structure during mitosis in *Drosophila melanogaster* embryos. *J. Cell Biol.* 94:463–470.

Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Jackson, M. R., T. Nilsson, and P. A. Peterson. 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3153–3162.

Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith. 1984. A short amino acid sequence able to specify nuclear localization. *Cell.* 39:499–509.

Klausner, R. D. 1989. Sorting and traffic in the central vacuolar system. *Cell.* 57:703–706.

Kornfeld, S., and I. Mellman. 1989. The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* 5:483–525.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the heads of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Liebhaber, S. A., and K. A. Begley. 1984. Structural and evolutionary analysis of the two chimpanzee alpha-globin mRNAs. *Nucleic Acids Res.* 11:8915–8929.

Loeb, J. A., and K. Drickamer. 1987. The chicken receptor for endocytosis of glycoproteins contains a cluster of N-acetylglucosamine-binding sites. *J. Biol. Chem.* 262:3022–3029.

Machamer, C. E., and J. K. Rose. 1987. A specific transmembrane domain of a coronavirus E1 glycoprotein is required for its retention in the Golgi region. *J. Cell Biol.* 105:1205–1214.

Meier, U. T., and G. Blobel. 1990. A nuclear localization signal binding protein in the nucleolus. *J. Cell Biol.* 111:2235–2245.

Meier, U. T., and G. Blobel. 1992. Nopp140 shuttles on tracks between the nucleolus and cytoplasm. *Cell.* 70:127–138.

Mellow, T. E., D. Halberg, and K. Drickamer. 1988. Endocytosis of *N*-acetylglucosamine-containing glycoproteins by rat fibroblasts expressing a single species of chicken liver glycoprotein receptor. *J. Biol. Chem.* 263: 5468–5473.

Moore, M. S., and G. Blobel. 1992. The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic factors. *Cell.* 69:939–950.

Muller, G. M., M. Shapira, and R. Arnon. 1982. Anti-influenza response achieved by immunization with a synthetic conjugate. *Proc. Natl. Acad. Sci. USA.* 79:569–573.

Nilsson, T., M. R. Jackson, and P. A. Peterson. 1989. Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the ER. *Cell.* 58:707–718.

Nilsson, T., J. M. Lucocq, D. Mackay, and G. Warren. 1991. The membrane spanning domain of β-1,4-galactosyltransferase specifies *trans* Golgi localization. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3567–3575.

Paabo, S., B. M. Bhat, W. S. M. Wold, and P. A. Peterson. 1987. A short

sequence in the COOH-terminus makes an adenovirus membrane glycoprotein a resident of the endoplasmic reticulum. *Cell.* 50:311–317.
Paine, P. L., L. C. Moore, and S. Horowitz. 1975. Nuclear envelope permeability. *Nature (Lond.).* 254:109–114.
Posnett, D. N., H. McGrath, and J. P. Tam. 1988. A novel method for producing anti-peptide antibodies. *J. Biol. Chem.* 263:1719–1725.
Robbins, J., S. M. Dilworth, R. A. Laskey, and C. Dingwall. 1991. Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell.* 64:615–623.
Rodriguez-Boulan, E., and J. Nelson. 1989. Morphogenesis of the polarized epithelial cell phenotype. *Science (Wash. DC).* 245:718–725.
Rothman, D., D. W. Andrews, M. C. Calajoy, and V. R. Lingappa. 1988. Construction of defined polytopic integral transmembrane proteins. The role of signal and stop transfer sequence permutations. *J. Biol. Chem.* 263:10470–10480.
Rothman, J. E., and L. Orci. 1992. Molecular dissection of the secretory pathway. *Nature (Lond.).* 355:409–415.
Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1987. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science (Wash. DC).* 239:487–491.
Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Senior, A., and L. Gerace. 1988. Integral membrane proteins specific to the inner nuclear membrane and associated with the nuclear lamina. *J. Cell Biol.* 107:2029–2036.
Silver, P. A. 1991. How proteins enter the nucleus. *Cell.* 64:489–497.
Simon, S. M., and G. Blobel. 1991. A protein-conducting channel in the endoplasmic reticulum. *Cell.* 65:371–380.
Suzuki, M. 1989. SPXX, a frequent sequence motif in gene regulatory proteins. *J. Mol. Biol.* 207:61–84.
Wong, S. H., S. H. Low, and W. Hong. 1992. The 17-residue transmembrane domain of β-galactoside alpha-2,6-sialyltransferase is sufficient for Golgi retension. *J. Cell Biol.* 117:245–258.
Worman, H. J., C. D. Evans, and G. Blobel. 1990. The lamin B receptor of the nuclear envelope inner membrane: a polytopic protein with eight potential transmembrane domains. *J. Cell Biol.* 111:1535–1542.
Worman, H. J., J. Yuan, G. Blobel, and S. D. Georgatos. 1988. A lamin B receptor in the nuclear envelope. *Proc. Natl. Acad. Sci. USA.* 85:8531–8534.