Abstract. We have examined transfected cells by immunofluorescence microscopy to determine the signals and structural features required for the targeting of integral membrane proteins to the inner nuclear membrane. Lamin B receptor (LBR) is a resident protein of the nuclear envelope inner membrane that has a nucleoplasmic, amino-terminal domain and a carboxyl-terminal domain with eight putative transmembrane segments. The amino-terminal domain of LBR can target both a cytosolic protein to the nucleus and a type II integral protein to the inner nuclear membrane. Neither a nuclear localization signal (NLS) of a soluble protein, nor full-length histone H1, can target an integral protein to the inner nuclear membrane although they can target cytosolic proteins to the nucleus. The addition of an NLS to a protein normally located in the inner nuclear membrane, however, does not inhibit its targeting. When the amino-terminal domain of LBR is increased in size from ~22.5 to ~70 kD, the chimeric protein cannot reach the inner nuclear membrane. The carboxyl-terminal domain of LBR, separated from the amino-terminal domain, also concentrates in the inner nuclear membrane, demonstrating two nonoverlapping targeting signals in this protein. Signals and structural features required for the inner nuclear membrane targeting of proteins are distinct from those involved in targeting soluble polypeptides to the nucleoplasm. The structure of the nucleocytoplasmic domain of an inner nuclear membrane protein also influences targeting, possibly because of size constraints dictated by the lateral channels of the nuclear pore complexes.

Integral membrane proteins synthesized on ER-bound ribosomes are partially translocated through protein-conducting channels and incorporated into the membrane with topologies determined by their primary structures (Blobel, 1980; Simon and Blobel, 1991). Many of these proteins reach the plasma membrane by vesicular transport in the secretory pathway (Palade, 1975; Rothman, 1994), which is modified in epithelial cells to provide for apical and basolateral membrane sorting (Rodriguez-Boulan and Nelson, 1989). Some of these integral membrane proteins, however, contain specific sequences that confer retention in or targeting to intermediate secretory compartments or organelles such as the ER, pre-Golgi vesicles, Golgi apparatus, lysosomes and endosomes (Nillson and Warren, 1994; Sandoval and Bakke, 1994; Schweizer et al., 1994).

Despite significant progress that has been made in deciphering the signals in integral membrane proteins responsible for their targeting to cytoplasmic compartments and organelles, little is known about the signals responsible for protein targeting to the membranes of the nuclear envelope. The nuclear envelope membranes are divided into three morphologically distinct but interconnected domains. The outer nuclear membrane has ribosomes on its cytoplasmic surface and is similar in composition to the RER with which it is directly continuous (Pathak et al., 1986). The inner nuclear membrane is associated on its nucleoplasmic face with the nuclear lamina, a meshwork of intermediate filament proteins called lamins (Aebi et al., 1986; Fisher et al., 1986; McKeon et al., 1986). Because the lamina is a discontinuous structure (Paddy et al., 1990), the inner nuclear membrane may also be directly associated with the chromatin at certain points. The third membrane domain of the nuclear envelope has been termed the pore membrane domain, which connects the inner and outer membranes at numerous points and is associated with the nuclear pore complexes (Hindshaw et al., 1992; Akey and Rademacher, 1993). Because the nuclear pore complexes are located at the junctures between the three nuclear envelope membrane domains, they may play an important role in establishing and maintaining the nonrandom segregation of integral proteins between these membranes.

Several integral membrane proteins that are nonrandomly localized in the membrane domains of the nuclear
envelope have been characterized. The outer nuclear membrane shares proteins with the RER (Amar-Costesec et al., 1974) and outer membrane proteins absent from the ER have not been identified. Unique proteins have been localized to the pore membrane domain that are most likely structural components of the nuclear pore complexes (Gerace et al., 1982; Hallberg et al., 1993; Wozniak et al., 1994). Several other integral membrane proteins have been identified that are present in the inner nuclear membrane and bind to components of the nuclear lamina and the chromatin (Worman et al., 1988, 1990; Senior and Gerace, 1988; Harel et al., 1989; Courvalin et al., 1990; Padan et al., 1990; Baier et al., 1991; Foisner and Gerace, 1993; Ye and Worman, 1994; Schuler et al., 1994). Only one vertebrate inner nuclear membrane protein termed LBR1, also referred to as lamin B receptor (Worman et al., 1988, 1990; Courvalin et al., 1990), p58 (Worman et al., 1988; Simos and Georgatos, 1992) and p54 (Baier et al., 1991), has been characterized by cDNA (Worman et al., 1990; Ye and Worman, 1994) and genomic (Schuler et al., 1994) cloning. LBR has a nucleoplasmic amino-terminal domain of 204 amino acids in chickens and 208 amino acids in humans followed by a hydrophobic, carboxy-terminal domain with eight putative transmembrane segments (Worman et al., 1990; Ye and Worman, 1994). The amino-terminal domain binds to DNA (Ye and Worman, 1994), B-type lamins (Worman et al., 1988; Smith and Blobel, 1994; Ye and Worman, 1994) and other nuclear proteins (Simos and Georgatos, 1992; Ye, Q. and H. J. Worman, manuscript submitted for publication) and is a substrate for phosphorylation by several nucleoplasmic protein kinases (Appelbaum et al., 1990; Worman et al., 1990; Courvalin et al., 1992; Simos and Georgatos, 1992).

As the membrane domains of the nuclear envelope are continuous with each other and the RER, integral proteins synthesized on ER-bound ribosomes can potentially reach all of the membrane domains by lateral diffusion in the interconnected proteolipid bilayers. Some viral integral membrane proteins that presumably lack specific targeting or retention signals are in fact found in the ER, outer, pore, and inner nuclear membranes after synthesis (Bergman and Singer, 1983; Torrisi and Bonnatti, 1985). Cellular proteins specifically localized to the various membrane domains of the nuclear envelope must therefore contain amino acid sequences, or other structural features, that confer targeting to and retention in their appropriate locations. For gp210, an integral membrane glycoprotein of the nuclear pore complex, both its single transmembrane segment and its cytoplasmic tail can confer localization to the pore membrane domain (Wozniak and Blobel, 1992). For LBR, its nucleoplasmic amino-terminal domain can target a nonnuclear type II integral membrane protein to the inner nuclear membrane (Soullam and Worman, 1993). In addition, the first transmembrane segment of LBR can mediate localization to the nuclear envelope (Smith and Blobel, 1993). Besides the results of these studies, however, little is known about protein targeting to and retention in the membrane domains of the nuclear envelope.

1. Abbreviations used in this paper: CHL, chicken hepatic lectin; CMPK, chicken muscle pyruvate kinase; HA, hemagglutinin; LBR, lamin B receptor; NLS, nuclear localization signal.

Plasmids and Methods

Plasmid Construction

Plasmids construct in pSVK3 (Pharmacia Fine Chemicals, Piscataway, NJ) that expressed chicken hepatic lectin (CHL), LBR, and the amino-terminal domain of LBR fused to the cytoplasmic side of the transmembrane segment of a truncated version of CHL were those described previously (Soullam and Worman, 1993). Plasmid p3PK, supplied by Dr. J. V. Frangioni (Harvard Medical School, Boston, MA), was used to express a truncated version of chicken muscle pyruvate kinase (CMPK) that lacked the first 16 amino acids (Frangioni and Neel, 1993). Other plasmids for protein expression in transfected cells were designed as described below. To produce some cDNA inserts for cloning, PCR was performed according to Saiki et al. (1987) using the GeneAmp Kit II (Perkin-Elmer Cetus Corp., Norwalk, CT). Parameters for PCR were denaturation for 1 min at 94°C, annealing for 2 min at 53°C, and extension for 3 min at 72°C. Custom oligonucleotides were obtained from Genset (Paris, France). Unless otherwise indicated, standard methods (Sambrook et al., 1989) were used for DNA purification, restriction endonuclease digestion, DNA ligation, bacterial transformation, and preparation of plasmid DNA.

Plasmids Encoding Soluble Chimeric Proteins with CMPK.

Plasmids encoding soluble chimeric proteins with CMPK were constructed using p3PK. Plasmid p3PK was digested with HindIII and BglIII and the desired cDNA inserts were inserted between these restriction endonuclease sites. The resulting plasmids encoded polypeptides fused to the amino-terminal side of CMPK lacking its first 16 amino acids.

To construct the plasmid encoding the amino-terminal domain of LBR fused to CMPK (AT LBR-CMPK), a PCR product from nucleotide +4 to +612 of chicken LBR cDNA, generated using LBR clone DJ5 (Worman et al., 1990) as the template, was cloned into p3PK. The sense PCR primer contained a HindIII site followed by the sequence ACATGTA to ensure translation initiation at its 5' end. The antisense primer contained a BamHI site at its 5' end. The amplified product was digested with HindIII and BamHI and cloned into p3PK between the HindIII and BgIII sites.

To construct the plasmid encoding histone H1 fused to CMPK (HIST-CMPK), a PCR product from nucleotide +1 to +678 of histone H1 cDNA, generated using a histone H1 genomic clone (supplied by Dr. N. Segil, The Rockefeller University, New York) as template, was cloned into p3PK. The sense PCR primer contained a HindIII site followed by the sequence ACCATG at its 5' end and the antisense primer contained a BglIII site at its 5' end. The amplified product was digested with HindIII and BgIII and cloned into p3PK between the HindIII and BgIII sites.

For the plasmid encoding the nuclear localization signal (NLS) of nucleoplasmin fused to CMPK (NU-CMPK), a PCR product encoding this sequence (Robbins et al., 1991), generated using a nucleoplasmin cDNA clone (supplied by Dr. C. Dingwall, Welcombe/CRC Institute, Cambridge, UK) as template, was cloned into p3PK. The sense PCR primer contained an NLS site followed by the sequence ACCATG at its 5' end and the antisense primer contained a BglIII site at its 5' end. The amplified product was digested with HindIII and BgIII and cloned into p3PK between these two sites.

To construct the plasmid encoding the NLS of SV-40 large T antigen fused to CMPK (SV-40 LG T-CMPK), two partially complementary oligonucleotides of sequences 5'-TAACATGCTTCCAAAGAAGCGAAGGTAAGGAGTTGAGAAGGTAAGGAGT-3' and 5'-GATCATGCTTCCAAAGAAGCGAAGGTAAGGAGTTGAGAAGGTAAGGAGT-3' were annealed at 37°C. The double-stranded product was treated with T4 polynucleotide kinase and cloned between the HindIII and BgIII sites of p3PK. The sense oligonucleotide encoded the sequence ACCATG followed by the NLS of SV-40 large T antigen (Kalderon et al., 1984).

Plasmids Encoding Designer Integral Membrane Proteins.

A cloning vector based on pSVK3 was constructed to express chimeric integral membrane proteins fused to a truncated version of CHL. To construct this cloning vector, a nine-amino acid influenza virus hemagglutinin (HA) tag tag
amino-terminal and carboxyl-terminal domains of LBR, the PCR product end followed by ACCATG for translation initiation. The antisense primer with EcoRI and XbaI.

EcoRI and Xbal and cloned into pSV-HA-CHL that was also digested had an XbaI site at its 5' end. The amplified product was digested with XbaI, was ligated into pSV-HA-CHL. The sense PCR primer contained an EcoRI site at its 5' end and the antisense primer a XbaI site at its 5' end. The amplified product was purified, digested with NheI and XbaI, and ligated into pSV-HA-AT LBR-CHL that was digested with XbaI (Nhel and XbaI sites are compatible for ligation). The orientation of the resulting plasmid, termed pSV-DUBLE AT LBR-CHL, was determined by digestion with several restriction enzymes. This plasmid expressed the fusion protein DBLE AT LBR-CHL. To produce the plasmid that encoded TPLE AT LBR-CHL, the PCR product from +4 to +612 of chicken LBR, amplified using the sense primer with the NheI site and antisense primer with the XbaI site at their respective 5' ends, was purified, digested with NheI and XbaI, and ligated into pSV-HA-AT LBR-CHL that was digested with XbaI.

Cell Culture and Transfection

COS-7 cells were grown in DME media containing 10% FBS, 50 U/ml penicillin, 50 mg/ml streptomycin, and 2 mM L-glutamine. Media and other reagents for cell culture were obtained from Gibco BRL (Gaithersburg, MD). For transfection, cells were grown to ~60% confluence on 60-mm diameter petri dishes and transfected with DNA 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 prior to preparation for immunofluorescence microscopy.

Antibodies Used in Immunofluorescence Microscopy

To detect CMPIK and CMPK fusion proteins, affinity-purified rabbit polyclonal antibodies (Frangioni and Neel, 1993) were supplied by Dr. J. V. Frangioni and used at a dilution of 1:150. Mouse monoclonal antibodies (clone R-7) against avian p54, which is identical or very similar to LBR (Leib and Drickamer, 1987) were supplied by Dr. K. Drickamer and used at a dilution of 1:300 to detect CHL. Polyclonal rabbit antibodies against vimonovirus HA tag were obtained from Berkeley Antibody Co. (Richmond, CA) and used at a dilution of 1:1,000. Fluorescence-conjugated anti-rabbit and anti-mouse immunoglobulin antibodies used as secondary antibodies for immunofluorescence microscopy were obtained from Jackson ImmunoResearch Labs., Inc. (West Grove, PA).

Immunofluorescence Microscopy and Digitonin Permeabilization

Transfected cells were grown to ~60% confluence on 35-mm diameter petri dishes, washed three times with PBS, fixed with 4% formaldehyde in PBS for 15 min, and then washed three times with PBS. Cells were permeabilized for 2 min with 0.5% Triton X-100 in PBS and washed three times with PBS containing 0.1% Tween-20 (Solution [Soln.] A). Cells were then incubated with PBS containing 0.1% Tween-20 and 2% BSA (Soln. B) for 30 min and then for 30 min with the desired antibody diluted in Soln. B. Cells were then washed five times with Soln. A, incubated for 30 min with a 1:300 dilution of the desired secondary antibody in Soln. B plus 2 µg/ml 4,6-diamidino-2-phenylindole, and then washed five times with Soln. A. Coverslips were mounted with a 2% wt/vol solution of 1,4-diazabicyclo(2,2,2)octane in 85% glycerol/15% 0.5 M Tris-HCl (pH 8.6). Immunofluo...
oressence microscopy was performed on either an Axiohot microscope (Carl Zeiss Inc., Thornwood, NY) or a laser scanning confocal microscope (TCS 4D; Leica Laserotechnik, Heidelberg, Germany) in the Brookdale Center for Molecular Biology at the Mount Sinai School of Medicine (New York). T-MAX 400 film (Eastman Kodak Co., Rochester, NY) was used for photography on the Axiohot microscope. Computerized images were printed on a digital continuous tone printer (XL7700; Eastman Kodak) when the confocal microscope was used.

Digitonin permeabilization of cells was performed essentially 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 grown as described above. After ~36 h in culture, one dish was prepared for immunofluorescence microscopy as usual, the other dish was washed three times with PBS, and the cells were permeabilized by incubation for 5 min at 4°C with 40 μg/ml of digitonin (Calbiochem-Novabiochem Corp., La Jolla, CA) in PBS. Digitonin was diluted into PBS from a 20 mg/ml stock in DMSO. After permeabilization with digitonin, cells were washed with PBS and prepared for immunofluorescence microscopy as usual, except that they were not fixed with formaldehyde, not permeabilized with Triton X-100, and Tween-20 was not present in SoI. A and SoN. B. Petri dishes derived from two or more different transfections were examined for each digitonin-permeabilization experiment.

Other Materials

Unless otherwise indicated, routine chemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Co. (Pittsburgh, PA). Enzymes for DNA cloning were obtained from New England Biolabs (Beverly, MA).

Results

Design and Construction of Proteins for Targeting Studies

To examine protein targeting to the inner nuclear membrane, COS-7 cells were transiently transfected with plasmids engineered to express various natural and designer proteins. As respective reference proteins for the inner nuclear membrane, cytoplasmic/plasma membranes and cytosol, plasmids were designed to express chicken LBR, CHL, and CMPK lacking its first 16 amino acids (Fig. 1a). Chicken LBR is an integral protein of the inner nuclear membrane with a basic, nucleoplasmic amino-terminal domain of 204 amino acids followed by a hydrophobic, carboxyl-terminal domain of 433 amino acids with eight putative transmembrane segments (Worman et al., 1990). CMPK is a type II integral membrane protein localized to the ER, endosomes, and plasma membrane that has an amino-terminal domain of 23 amino acids that faces the cytoplasm, a single transmembrane segment and a luminal, carboxyl-terminal domain of 160 amino acids (Chiacchia and Drickamer, 1984; Mellow et al., 1988). The truncated version of CMPK is a completely soluble, normally cytosolic protein of ~50 kDa (Frangioni and Neel, 1993).

Several novel, nonmembrane proteins were designed for expression in transfected cells (Fig. 1b). One was the amino-terminal domain of LBR separate from the carboxyl-terminal domain (AT LBR). Others were the amino-terminal domain of LBR fused to truncated CMPK (AT LBR-CMPK) and histone H1, a nuclear protein of similar size and charge, fused to truncated CMPK (HIST-CMPK). Plasmids were also constructed to express truncated CMPK with the NLS of nucleoplasmin (NU-CMPK) and the NLS of SV-40 large T antigen (SV-40 LG T-CMPK) at its amino terminus.

Several truncated and chimeric integral membrane proteins were also designed for these studies (Fig. 1c). One was the amino-terminal domain of LBR fused to the cytoplasmic side of the transmembrane segment of a truncated

Figure 1. Schematic diagrams of proteins expressed in transfection experiments. (a) Native proteins expressed in transfected cells were chicken LBR (LBR), a truncated version of chicken muscle pyruvate kinase which lacked the first 16 amino acids (CMPK), and chicken hepatic lectin (CHL). The nucleoplasmic, amino-terminal domain of LBR is represented by vertical stripes and the hydrophobic, carboxyl-terminal domain is represented by horizontal stripes. CMPK is represented by thin diagonal stripes with a right to left orientation. The single transmembrane segment of CHL is black and is preceded by the 23-amino acid cytoplasmic amino-terminal domain and followed by the 160-amino acid luminal, carboxyl-terminal domain which is gray. The amino-terminus of each protein is shown at the left. (b) Truncated and chimeric soluble proteins expressed in transfected cells were the amino-terminal domain of LBR separate from the carboxyl-terminal domain (AT LBR), the amino-terminal domain of LBR fused to truncated CMPK (AT LBR-CMPK), histone H1 (thick diagonal stripes with left to right orientation) fused to CMPK (HIST-CMPK), the NLS of nucleoplasmin fused to CMPK (NU-CMPK), and the NLS of SV-40 large T antigen fused to CMPK (SV-40 LG T-CMPK). The amino terminus of each protein is shown at the left. (c) Truncated and chimeric integral membrane proteins expressed in transfected cells were the amino-terminal domain of LBR fused to the cytoplasmic side of the transmembrane segment of a truncated version of CHL from amino acid 23 to amino acid 131 (AT LBR-CHL), histone H1 fused to truncated CHL (HIST-CHL), the NLS of nucleoplasmin fused to truncated CHL (NU-CHL), the NLS of SV-40 large T antigen fused to truncated CHL (SV-40 LG T-CHL). Proteins were also expressed with the amino-terminal domain of LBR inserted between the nucleoplasmin NLS (NU-AT LBR-CHL) and the SV-40 large T antigen NLS (SV-40 LG T-CHL) and truncated CHL. The hydrophobic, carboxyl-terminal domain of LBR from amino acid 201 to amino acid 637 (CT LBR) was also expressed. Other chimeric proteins were CMPK inserted between the amino-terminal domain and transmembrane segment of truncated CHL (AT LBR-CMPK-CHL), CMPK inserted between the amino-terminal domain and the carboxyl-terminal domain of LBR and two (DBLE AT LBR-CHL) and three (TPLE AT LBR-CHL) tandem repeats of the amino-terminal domain of LBR fused to the transmembrane segment of truncated CHL. HIST-CHL, NU-CHL, SV-40 LG T-CHL, NU-AT LBR-CHL, SV-40 LG T-AT LBR-CHL, and CT LBR each contained a nine-amino acid influenza virus HA epitope tag at their amino termini. The carboxyl-terminal domain of LBR is not drawn to scale in the chimeric proteins. The amino terminus of each protein is shown at the left.
version of CHL that contained the transmembrane segment and the luminal domain up to amino acid 131 (AT LBR-CHL). Three others were histone H1 (HIST-CHL), the NLS of nucleoplasmin (NU-CHL) and the NLS of SV-40 large T antigen (SV-40 LG T-CHL) each fused to the transmembrane segment of truncated CHL. These three chimeric proteins also had a nine-amino acid HA tag at their amino termini. Chimeric proteins similar to NU-CHL and SV-40 LG T-CHL were also expressed that contained the amino-terminal domain of LBR inserted in-frame between the nucleoplasmin NLS and the transmembrane segment of CHL (NU-AT LBR-CHL) and between the SV-40 large T antigen NLS and the transmembrane segment of CHL (SV-40 LG T-AT LBR-CHL). Another protein was the carboxyl-terminal domain of LBR with its amino-terminal domain replaced by a nine-amino acid HA tag (CT LBR). Another designer protein contained CMPK inserted between the amino-terminal domain of LBR inserted in-frame between the nucleoplasmin NLS and the transmembrane segment of CHL (NU-AT LBR-CHL) and another contained CMPK inserted between the amino-terminal and carboxyl-terminal domains of LBR (AT LBR-CMPK-CT LBR). Proteins were also designed with two (DBLE AT LBR-CHL) and three (TPLE AT LBR-CHL) tandem copies of the LBR amino-terminal domain fused to the cytoplasmic side of the transmembrane segment of CHL.

Cellular Compartments Determined by Immunofluorescence Microscopy

LBR, CHL, and CMPK were used, respectively, as markers for the inner nuclear membrane, cytoplasmic/plasma membranes, and cytosol in transfected COS-7 cells. When examined by immunofluorescence microscopy with antibodies that recognized chicken LBR but not its mammalian homologue, smooth nuclear rim fluorescence was seen in transfected cells that expressed LBR consistent with its localization in the inner nuclear membrane (Fig. 2 a). In contrast, strong labeling of the ER and other cytoplasmic membranes, along with weaker labeling of the plasma membrane, was seen in transfected cells that expressed CHL (Fig. 2 b). The fluorescence enhancement at the nuclear periphery in cells that expressed CHL was consistent with labeling of the outer nuclear membrane and ER as well as the inner nuclear membrane. Nevertheless, exclusive labeling of the inner nuclear membrane (Fig. 2 a) was easily distinguishable from labeling of the outer nuclear and ER membranes (Fig. 2 b). Inner nuclear membrane localization was also confirmed by epitope accessibility experiments in digitonin-permeabilized cells (see below). The nucleoplasmic compartment was also easily distinguished from the cytosolic compartment of cells by immunofluorescence microscopy. The DNA-binding dye 4,6-diamidino-2-phenylindole gave distinct fluorescence labeling of the nucleoplasm (Fig. 2 c) while antibodies against CMPK clearly labeled the cytosol in cells transfected to express the truncated version of this protein (Fig. 2 d).

LBR Amino-Terminal Domain Can Target a Cytosolic Protein to the Nucleus

As previously demonstrated (Soullam and Worman, 1993; Smith and Blobel, 1993), the amino-terminal domain of LBR separate from its carboxyl-terminal domain concentrated in the nucleoplasm of transfected COS-7 cells (Fig. 3 a). This domain of LBR is only ~22.5 kD and could reach the nucleoplasm by diffusion and concentrate there by binding to nuclear ligands. To determine if the amino-terminal domain of LBR can mediate active nuclear transport, we fused it to the truncated version of CMPK, a normally cytosolic protein of ~50 kD (Frangioni and Neel, 1993). This chimeric protein of more than 70 kD, which is too large to enter the nucleus by diffusion (Paine et al., 1975), is concentrated in the nucleoplasm (Fig. 3 b). Histone H1, which is similar in overall size and charge to the amino-terminal domain of LBR, contains a functional NLS (Breeuwer and Goldfarb, 1990) and has DNA binding activity, was also fused to CMPK and the chimeric protein concentrated in the nucleoplasm (Fig. 3 c). The bipartite NLS of nucleoplasm (Fig. 3 d) and the NLS of SV-40 large T antigen (Fig. 3 e) also targeted CMPK to the nucleus whereas truncated CMPK was cytosolic (Fig. 3 f). These results demonstrated that the amino-terminal do-
main of LBR contains a functional NLS that can target a soluble, cytosolic protein to the nucleus.

**The Amino-Terminal Domain of LBR Can Target an Integral Protein to the Inner Nuclear Membrane but NLSs of Nonmembrane Proteins Cannot**

We have previously shown that the amino-terminal domain of LBR fused to the cytoplasmic side of the transmembrane segment of a type II integral membrane protein is targeted to the inner nuclear membrane (Soullam and Worman, 1993). Because this domain of LBR can also target soluble proteins to the nucleus as histone H1, the NLS of SV-40 large T antigen and the NLS of nucleoplasmin can, we determined if these polypeptides can similarly target a type II integral membrane protein to the inner nuclear membrane. When the amino-terminal domain of LBR was fused to the cytoplasmic side of the transmembrane segment of a truncated CHL, nuclear rim fluorescence was observed in transfected cells examined by immunofluorescence microscopy (Fig. 4 a). When histone H1 (Fig. 4 b), the NLS of nucleoplasmin (Fig. 4 c) or the NLS of SV-40 large T antigen (Fig. 4 d) were similarly fused to truncated CHL, however, labeling of cytoplasmic/plasma membranes was observed in a pattern identical to that of native CHL. To confirm that the proteins with histone H1 and the NLSs of SV-40 and nucleoplasmin were correctly synthesized in transfected cells, immunofluorescence was also performed using antibodies against CHL. These antibodies recognized the carboxyl-terminal regions of the chimeric proteins whereas the antibodies against HA recognized the amino-termini. Identical labeling was observed with both antibodies, suggesting that the expressed polypeptides were correctly synthesized in transfected cells.

As the recognized epitopes of these proteins were localized on the nucleoplasmic or cytoplasmic sides of intracellular membranes, we used selective permeabilization of the plasma membrane with digitonin to determine if they were located on the inner aspect of the nuclear envelope versus outer nuclear, pore, or other cytoplasmic membranes. Under appropriate conditions, digitonin selectively permeabilizes the plasma membrane such that in unfixed, digitonin-permeabilized cells, antigenic epitopes facing the nuclear pore complexes and cytoplasm are accessible to antibodies whereas epitopes located inside the nucleus are not accessible (Adam et al., 1990). When unfixed, digitonin-permeabilized cells transfected to express the amino-terminal domain of LBR fused to CHL were examined by immunofluorescence microscopy in five different experiments, labeling of transfected cells was never observed (Fig. 4 e) as it was in fixed cells permeabilized as usual with Triton X-100. The recognized epitopes were therefore inaccessible to antibodies and located inside the nucleus. In contrast, labeling similar to that seen in fixed cells permeabilized with Triton X-100 was seen in unfixed cells permeabilized with digitonin that expressed histone H1 (Fig. 4 b), the NLS of nucleoplasmin (Fig. 4 e) and the SV-40 large T antigen NLS (Fig. 4 d) fused to CHL. The results confirmed the correct topologies of these chimeric integral membrane proteins and showed that the LBR amino-terminal domain fusion protein was localized almost solely in the inner nuclear membrane while the other three proteins were localized primarily in the outer nuclear, cytoplasmic and possibly the nuclear pore membranes.

The subcellular localizations of these chimeric integral membrane proteins were also confirmed by confocal laser scanning immunofluorescence microscopy (Fig. 5). LBR was found exclusively localized to the nuclear envelope in serial optical confocal sections, a representative section of which is shown (Fig. 5 a). The chimeric protein with the amino-terminal domain of LBR fused to CHL was also localized essentially only at the nuclear periphery (Fig. 5 b). In contrast, the chimeric proteins with histone H1 (Fig. 5 c), the NLS of nucleoplasmin (Fig. 5 d) and the NLS of SV-40 large T antigen (Fig. 5 e) fused to CHL were present in the ER and other cytoplasmic membranes in all optical sections.
Figure 4. Cellular localization of chimeric integral membrane proteins determined by immunofluorescence microscopy in transfected cells permeabilized with Triton X-100 or permeabilized with digitonin. In a-d, transfected COS-7 cells were permeabilized with Triton X-100 and fixed for immunofluorescence microscopy as usual. In e-h, COS-7 cells, transfected on the same petri dishes as those in the panels immediately above, were permeabilized with digitonin at 4°C and not fixed before antibody labeling and immunofluorescence microscopy. The expressed chimeric proteins were the amino-terminal domain of LBR fused to a truncated version of CHL (a and e) and histone H1 (b and f), the NLS of nucleoplasmin (c and g) and the NLS of SV-40 large T antigen (d and h) each similarly fused to truncated CHL. In a and e, antibodies against the amino-terminal domain of LBR were used for labeling. In b-d and f-h, antibodies against HA were used. Bars: (a-d) and (e-h) 5 μm.

In summary, NLSs of soluble nuclear proteins are not sufficient for an integral membrane protein to be targeted to and retained in the inner nuclear membrane. A DNA binding protein that contains an NLS, such as histone H1, also cannot necessarily target a transmembrane protein to this location. The amino-terminal domain of LBR must therefore contain special features that can mediate targeting to and retention in the inner nuclear membrane.

The NLS of a Soluble Nuclear Protein Does Not Inhibit Targeting to the Inner Nuclear Membrane

The nucleoplasmic, amino-terminal domain of LBR can target an integral protein to the inner nuclear membrane but NLSs of soluble proteins cannot. However, the amino-terminal domain of LBR can function as an NLS and target a large soluble protein to the nucleoplasm and contains two stretches that resemble the bipartite NLS of nucleoplasmin. It can therefore be inferred that NLSs do not interfere with the ability of a protein to reach the inner nuclear membrane. To confirm this, we made constructs that expressed a polypeptide normally localized to the amino-terminal domain of LBR fused to CHL. In another, the NLS of SV-40 large T antigen was placed at the amino terminus of the same chimeric polypeptide. Both of these NLS-containing polypeptides were targeted to the nuclear envelope as demonstrated by nuclear rim fluorescence seen on confocal immunofluorescence microscopy (Fig. 6). Epitope accessibility experiments in digitonin-permeabilized cells confirmed that these polypeptides were localized to the inner nuclear membrane (data not shown). These experiments demonstrated that the addition of a well-characterized NLS to the nucleocytoplasmic domain of an integral membrane protein normally localized to the inner nuclear membrane does not inhibit the ability of such a protein to reach this location.

Size of the Cytoplasmic Domain Affects Inner Nuclear Membrane Targeting

The nuclear pore complexes contain a central channel through which soluble proteins are thought to be actively transported as well as lateral channels of 10-nm diameter through which proteins <40–50 kD can presumably diffuse (Hindshaw et al., 1992; Akey and Radermacher,
Because the lateral channels are located near the pore membrane, it is likely that the nucleocytoplasmic domain of a protein transported to the inner nuclear membrane must fit through these channels. We therefore hypothesized that proteins with large nucleocytoplasmic domains would not be able to reach efficiently the inner nuclear membrane unless they can fit through the lateral channels of the pore complexes. To test this hypothesis, we enlarged the nucleoplasmic domains of proteins normally localized in the inner nuclear membrane.

While the amino-terminal domain of LBR fused to truncated CHL was able to concentrate in the inner nuclear membrane (Fig. 7 a), a chimeric protein in which the ~50-kD protein CMPK was inserted between these two polypeptides did not concentrate in the inner nuclear membrane (Fig. 7 b). Similarly, insertion of CMPK between the nucleoplasmic, amino-terminal domain and the first transmembrane segment of the carboxyl-terminal domain of LBR prevented localization of the protein in the inner nuclear membrane (Fig. 7 c). These localizations were confirmed by epitope accessibility experiments in digitonin-permeabilized cells (data not shown). Furthermore, identical results were obtained with antibodies that recognized the amino-terminal domain of LBR and antibodies against CMPK and CHL suggesting that the chimeric polypeptides were appropriately synthesized and not degraded in transfected cells.

We further confirmed that the size of a protein's nucleocytoplasmic domain alters its ability to be efficiently transported to the inner nuclear membrane by fusing tandem copies of the amino-terminal domain of LBR (~22.5 kD) to truncated CHL. Polypeptides with one (Fig. 8 a) or two (Fig. 8 b) LBR amino-terminal domains fused to the cytoplasmic side of the transmembrane segment of CHL were not concentrated in the inner nuclear membrane (Fig. 8 c). These localizations were confirmed by epitope accessibility experiments in digitonin-permeabilized cells (data not shown). Furthermore, identical results were obtained with antibodies against CMPK and CHL suggesting that the chimeric polypeptides were appropriately synthesized and not degraded in transfected cells.

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able to reach efficiently the inner nuclear membrane. In contrast, a chimeric protein with three tandem copies of the amino-terminal domain of LBR fused to CHL was primarily retained in the ER and cytoplasmic membranes (Fig. 8c). Inner nuclear membrane localization of the first two of these polypeptides was confirmed by epitope accessibility in digitonin-permeabilized transfected cells (data not shown).

These results show that an intergral membrane protein with a nucleocytoplasmic domain as large as ~45 kD can concentrate in the inner nuclear membrane. When the size of the nucleocytoplasmic domain is increased to ~70 kD, the protein can no longer efficiently concentrate in the inner nuclear membrane, despite containing sufficient targeting signals.

**LBR Contains Two Nonoverlapping Inner Nuclear Membrane Targeting Signals**

When the amino-terminal domain of LBR was replaced by the cytosolic protein α-globin, the fusion protein was largely retained in the ER and other cytoplasmic membranes (Soullam and Worman, 1993). It has also been shown that the transmembrane segments of LBR, lacking the amino-terminal domain, can efficiently accumulate in the inner nuclear membrane (Smith and Blobel, 1993). We confirmed that LBR contains a second inner nuclear membrane targeting signal within its carboxyl-terminal domain by expressing this domain with only a nine amino acid HA tag at its amino terminus in transfected COS-7 cells. Nuclear rim fluorescence was seen in transfected cells that expressed chicken LBR, the amino-terminal domain of LBR fused to truncated CHL and the carboxyl-terminal domain of LBR with the HA tag (Fig. 9a–c). Inner nuclear membrane localization of these expressed proteins was confirmed by permeabilization with digitonin (Fig. 9d–f). These results demonstrated that LBR contains two nonoverlapping targeting signals. The carboxyl-terminal domain targeting element did not function when α-globin was fused to it (Soullam and Worman, 1993), possibly as a result of α-globin aggregation that occurs in the absence of β- or δ-globin.

**Discussion**

**Mechanism of Inner Nuclear Membrane Targeting**

The results of the present study show that structural motifs different from those sufficient to target soluble proteins to
the nucleus are needed to target integral proteins to the inner nuclear membrane. The nucleoplasmic, amino-terminal domain of inner nuclear membrane protein LBR can function as an NLS when attached to a soluble cytosolic protein and as an inner nuclear membrane targeting signal when attached to the cytoplasmic side of a transmembrane segment. With regard to its ability to function as an NLS for soluble proteins, this polypeptide has two sequences similar to the bipartite NLS of nucleoplasmin (Worman et al., 1990; Soullam and Worman, 1993). The well-characterized NLSs of SV-40 large T antigen (Kalderon et al., 1994) and nucleoplasmin (Robbins et al., 1991); however, cannot target an integral protein to the inner nuclear membrane, demonstrating that such sequences alone are not sufficient to mediate this targeting. However, when such sequences are present in the nucleoplasmic domain of an inner nuclear membrane protein, targeting is not inhibited.

The amino-terminal domain of LBR has the capacity to bind to DNA and B-type lamins (Ye and Worman, 1994). For nonmembrane proteins, both an NLS for active nuclear uptake and an appropriate binding domain for a nuclear component are necessary to prevent export from the nucleus and shuttling between the nucleoplasm and cytoplasm (Schmidt-Zachmann et al., 1993). Therefore, if the strength of an NLS were not sufficient to overcome the rate of export from the nucleus, nuclear accumulation...
would not be observed. A combination of an NLS and nuclear ligand binding activity, however, does not appear to be sufficient for targeting to and retention in the inner nuclear membrane because histone H1, which is actively transported to the nucleoplasm (Breeuwer and Goldfarb, 1990) and has DNA binding capacity, cannot accumulate in the inner nuclear membrane when fused to the cytoplasmic side of a transmembrane segment. The amino-terminal domain of LBR must therefore have different properties than histone H1 to be able to mediate targeting to and retention in the inner nuclear membrane.

The simplest mechanism for inner nuclear membrane targeting and retention consistent with all of the results of the present study, nonetheless, is that integral proteins reach this location by lateral diffusion in the interconnected membranes of the RER and nuclear envelope and concentrate there by binding to nuclear ligands. A necessary structural feature, however, would be that the nucleocytoplasmic domains of such proteins must fit through the lateral channels of the nuclear pore complex to be efficiently targeted in interphase. Consistent with this is the finding that LBR no longer concentrates in the inner nuclear membrane when its amino-terminal domain is artificially enlarged. Although similar in size and charge to the amino-terminal domain of LBR, histone H1 may not be able to pass through the lateral channels of the pore complexes for other reasons. One possibility is that histone H1 adopts an unusual tertiary structure or binds to other cytosolic proteins that prevent transit through the lateral channels. Alternatively, histone H1 fused to CHL may be able to reach the inner nuclear membrane but not be retained there because it no longer has DNA binding capacity in the context of being fused to a transmembrane segment. Complex formation of histone H1 with cytosolic NLS-binding proteins is not likely to be responsible for blocking transport through the lateral channel of the pore complexes as other integral membrane proteins with NLSs in their nucleocytoplasmic domains can concentrate in the inner nuclear membrane. Cytosolic NLS-binding proteins therefore may not recognize NLSs that are fused to transmembrane segments, or alternatively, may have a rate constant for association with NLSs that is lower than that for diffusion from the RER to the inner nuclear membrane. It is also possible that such proteins dissociate from NLSs when they reach the vicinity of the nuclear pore complex.

After diffusing through the lateral channels of the nuclear pore complexes, resident integral membrane proteins can be retained in the inner nuclear membrane by binding to nuclear ligands. Some viral integral membrane proteins that presumably lack any specific targeting signals, such as the vesicular stomatitis virus G protein, that has a cytoplasmic domain of ~3 kD (Katz et al., 1977), can reach the inner nuclear membrane (Bergman and Singer, 1983), but are also found in many different cellular membranes. This finding suggests that, although integral proteins of appropriate structure can reach the inner nuclear membrane by lateral diffusion from the RER, nuclear ligand binding is required for retention and a unique inner nuclear membrane localization. Alternatively, integral membrane proteins may dimerize with resident proteins already in the inner nuclear membrane via their respective transmembrane segments and be retained there (Smith and Blobel, 1993).

Although our findings are consistent with the hypothesis that the predominant pathway for the movement of membrane proteins from the RER to the inner nuclear membrane during interphase is through the pore membrane domain, it is possible that alternative pathways exist. One possibility is that some nuclear pore complexes are unstable in interphase and that breakdown of pore complexes results in fusion between the inner and outer nuclear membranes, providing a pathway for protein diffusion that bypasses the pore membrane. It is also possible that the size limitations imposed by the lateral channels of the pore complexes can be overcome if a protein is proteolytically truncated to a smaller size before diffusion in the pore membrane domain. It is also possible that transcytosis of membrane vesicles across the perinuclear space can account for some movement between the inner and outer membrane domains of the nuclear envelope, although this is not generally observed in cells. The existence of such alternative pathways can explain the finding that some integral membrane proteins with large cytoplasmic domains can to some extent reach the inner nuclear membrane (Smith and Blobel, 1993).

In addition to targeting during interphase, sorting to and retention in the inner nuclear membrane could occur during mitosis (see below). If only mitotic sorting occurred, proteins would be seen in the RER/outer nuclear membrane before mitosis and only be present in the inner nuclear membrane after mitosis. For all of the polypeptides studied in the present report, this mixed distribution of expressed polypeptides was never observed and labeling patterns were identical for cell cultures grown for 16–48 h after transfection. Proteins localized to the inner nuclear membrane were observed in that location both in individual transfected cells and in couples or groups of cells that likely arose from mitotic divisions of a single transfected cell. Therefore mitotic sorting was not the sole operative mechanism for targeting proteins to the inner nuclear membrane in these studies. It could, however, hypothetically provide a pathway for some proteins to reach the inner nuclear membrane that cannot be targeted to that location during interphase (see also below).

**Implications of Studies on LBR for the Targeting of Other Integral Membrane Proteins**

Several other proteins have been localized to the inner nuclear membrane in addition to LBR. At the present time, the primary structure of only one these, otefin from *Drosophila melanogaster*, has been reported in the literature (Padan et al., 1990). *Drosophila* otefin is a mostly hydrophilic protein of ~45 kD with a single putative transmembrane segment at its carboxyl terminus. Similar to LBR, otefin does not have a cleaved amino-terminal signal sequence and its hydrophilic amino-terminal domain likely faces the cytoplasm when synthesized on ER-bound ribosomes and the nucleus when in the inner nuclear membrane. It has been hypothesized, although not experimentally demonstrated, that the hydrophilic amino-terminal domain of otefin interacts with lamins and/or chromatin components (Padan et al., 1990). The nucleocytoplasmic
LBR Contains Two Nonoverlapping Inner Nuclear Membrane Targeting Signals

We have confirmed the results of two previous studies showing that LBR contains two nonoverlapping inner nuclear membrane targeting signals (Soullam and Worman, 1993; Smith and Blobel, 1993). Therefore, in native LBR, two independent targeting signals, the amino-terminal domain (Soullam and Worman, 1993) and the first transmembrane segment (Smith and Blobel, 1993), may function in targeting to the nuclear envelope and sorting to the inner nuclear membrane. The presence of two nonoverlapping targeting signals is not unprecedented as a similar situation has been found for protein targeting to the membranes of the Golgi apparatus. As elegantly reviewed by Nilsson and Warren (1994), proteins localized to the membranes of the Golgi are generally retained there by their transmembrane segments while retrieval to these membranes is mediated by sequences in their cytoplasmic tails.

Two nonoverlapping sequences that can independently mediate targeting to the trans-Golgi membranes are present in the transmembrane segment and cytoplasmic domain of the integral membrane glycoprotein TGN38 (Ponnambalam et al., 1994). Localization of Sed5 to the cis-Golgi membranes also can be mediated by either the transmembrane segment or cytoplasmic domain of this protein (Banfield et al., 1994). In addition, both the single transmembrane segment and the cytoplasmic domain have been shown to mediate targeting to the nuclear envelope (Worman and Blobel, 1992). It therefore appears that the presence of nonoverlapping targeting signals in intracellular integral membrane proteins may be a common occurrence that ensures appropriate targeting, retention, and retrieval. Transmembrane segments may also mediate targeting by mechanisms such as homo-oligomerization or differential retention in membranes of various thicknesses (Bretschner and Munro, 1993; Nilsson and Warren, 1994). Cytoplasmic domains likely mediate targeting by binding to other proteins, or in the case of inner nuclear membrane proteins, possibly nucleic acids.

For targeting to the Golgi apparatus, the transmembrane segments of integral proteins mediate retention in certain membranes as the proteins proceed along the secretory pathway, while the cytoplasmic domains function in the retrieval of membrane vesicles that contain these proteins but have advanced beyond their usual location in the secretory pathway (Nilsson and Warren, 1994). The amino-terminal domain of LBR may also play a functional role analogous to that of retrieval of proteins in the secretory pathway. During nuclear envelope reassembly at the end of mitosis, inner nuclear membrane-derived vesicles that contain LBR are targeted to decondensing chromosomes before most other nuclear envelope components (Chaudhary and Courvalin, 1993). As the amino-terminal domain of LBR binds to components of chromatin (Ye and Worman, 1994; Ye, Q., and H. J. Worman, manuscript submitted for publication), this domain may mediate the targeting of inner nuclear membrane-derived vesicles to decondensing chromosomes. The cell cycle–dependent phosphorylation of specific amino acid residues in LBR (Courvalin et al., 1992) may even alter its interactions with chromatin components at different times in mitosis. Both the amino-terminal domain and first transmembrane segment of LBR may therefore function in targeting to and retention in the inner nuclear membrane after protein synthesis in interphase. However, only the amino-terminal domain may function in the targeting/retrieval of inner nuclear membrane-derived vesicles to decondensing chromosomes at the end of mitosis.

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