

A Visual Screen of a GFP-fusion Library Identifies a New Type of Nuclear Envelope Membrane Protein

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Abstract. The nuclear envelope (NE) is a distinct sub-domain of the ER, but few membrane components have been described that are specific to it. We performed a visual screen in tissue culture cells to identify proteins targeted to the NE. This approach does not require assumptions about the nature of the association with the NE or the physical separation of NE and ER. We confirmed that screening a library of fusions to the green fluorescent protein can be used to identify proteins targeted to various subcompartments of mammalian cells, including the NE. With this approach, we

identified a new NE membrane protein, named nurim. Nurim is a multispansing membrane protein without large hydrophilic domains that is very tightly associated with the nucleus. Unlike the known NE membrane proteins, it is neither associated with nuclear pores, nor targeted like lamin-associated membrane proteins. Thus, nurim is a new type of NE membrane protein that is localized to the NE by a distinct mechanism.

Key words: nuclear envelope • nurim • green fluorescent protein • protein targeting • visual screen

THE nuclear envelope (NE)¹ is structurally a prominent domain of the ER. It consists of two lipid bilayers, the inner and outer nuclear membranes, which are joined at nuclear pores. The inner membrane is supported by the lamina, a network of intermediate filament proteins (41), whereas the outer membrane is connected with the peripheral ER. Given that the inner and outer nuclear membranes and the peripheral ER are diffusionally continuous (4, 42), proteins that reside in the NE must be specifically targeted to it. Identifying these proteins is crucial to understand how they are segregated away from the connected peripheral ER and, ultimately, what functions are localized to the NE.

Very few membrane proteins of the NE have been identified. These proteins can be grouped into two classes, one associated with the nuclear pores and the other directly associated with lamins. The two known nuclear pore mem-

brane proteins, gp210 and POM121, are topologically quite different from one another, and are suggested either to anchor pores in the membrane or to anchor other components to the pores (3). Regions of each protein required for localizing it to pores have been identified (38, 44), but it is not clear what targets the nuclear pore to the nuclear envelope.

The lamin-associated class of NE membrane proteins has five members: the lamin B receptor (LBR), lamina-associated polypeptide 1 (LAP1, with A, B, and C isoforms), lamina-associated polypeptide 2 (LAP2), emerin, and MAN1. At the primary structure level, each of these proteins begins with a domain of at least 200 amino acids that is followed by a variable number of transmembrane domains. The NH₂-terminal domain extends into the nucleoplasm and is, thus, available to bind nuclear components. Lamin-binding has been directly shown for LBR (43), LAP1, and LAP2 (13). Emerin (12, 26) and MAN1 (Lin, F., D. Blake, I. Callebaut, M. McBurney, M. Paulin-Levasseur, and H.J. Worman. 1998. American Society for Cell Biology Annual Meeting. 2595 (abstr.)) exhibit behavioral and sequence similarity with LAP2, but have not been tested for lamin-binding. The NE targeting signals have been shown to reside in the nucleoplasmic domains of LBR (39), LAP2 (15), and emerin (12). Whereas LAP2 and LBR have been shown to bind chromatin (13, 47) as well as lamins, for LAP2 only the lamin-binding determinants are required for NE targeting (16). The nucleoplasmic domains of LBR, LAP2, and emerin are sufficient to

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1. *Abbreviations used in this paper:* CFP, cyan fluorescent protein; EST, expressed sequence tag; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; HO-2, heme oxygenase-2; LAP1 and -2, lamina-associated polypeptide 1 and -2; LBR, lamin B receptor; NE, nuclear envelope; TX-100, Triton X-100; VLP, visually localized protein; YFP, yellow fluorescent protein.

target other membrane proteins to the NE (16, 30, 39). It is likely that all these proteins are initially integrated into the peripheral ER, and then diffuse laterally in the lipid bilayer until they are retained in the inner nuclear membrane by binding to lamins. This mechanism is supported by measurements of the diffusional mobility in the peripheral ER and NE of fusion proteins consisting of LBR or emerlin and green fluorescent protein (GFP): in the peripheral ER they are very mobile, but in the NE their mobility is drastically reduced (11, 30). Other support comes from experiments with fused cells in which LAP1C moved from one nucleus to another, but accumulated only in nuclei containing lamin A/C (31). The lamin-binding class of proteins, thus, shares three interrelated features: a long NH₂-terminal nucleoplasmic domain, the ability to bind lamins, and a common targeting mechanism to the NE.

We considered it unlikely that the nuclear pore- and lamin-associated proteins are the only proteins that distinguish the NE from the peripheral ER and, therefore, wished to identify additional proteins segregated to the NE. We chose a visual screening method because it does not require physical separation of the connected NE and ER structures or assumptions about the types of interactions that associate proteins with those structures. Visual screens for localized proteins have been described previously in *Saccharomyces cerevisiae* (7) and *Schizosaccharomyces pombe* (32) but not higher eukaryotic cells. For *S. cerevisiae*, individual strains with random insertions of LacZ into the genome were screened by immunofluorescence using a β -galactosidase antibody. Although the approach was not used specifically to identify novel proteins, it demonstrated that strains with β -galactosidase fusion proteins localized to a variety of subcellular structures could be identified. Similarly, GFP fusion proteins targeted to specific nuclear regions were visualized in *S. pombe*. In this screen, strains harbored plasmids from a library of random genomic DNA fragments fused to the GFP coding sequence (32). Since fusion proteins localized to a variety of intracellular compartments in yeast, we believed that targeted fusion proteins should be identifiable in mammalian cells, which have more distinct subcellular structure than yeast at the light microscope level.

To implement a visual screen in cells with more complex genomes, we used a pooled GFP-cDNA expression library, screened transiently transfected cells for particular fluorescence patterns, and isolated clones by repeated rounds of screening and subdivision of pools of clones. We demonstrate that proteins localized to a variety of subcellular structures can be identified by a visual screen in higher eukaryotes. In many cases the GFP fusion protein was targeted to the same structure as the endogenous protein. Using this method we have identified a membrane protein of the NE, nurim, which belongs to neither the nuclear pore- nor lamin-associated class and appears to be targeted to the NE by a novel mechanism.

Materials and Methods

Construction of a GFP-cDNA Fusion Library

The human osteosarcoma cell line U2OS (HTB-96; American Type Culture Collection) was used as the RNA source for library construction. In

brief, mRNA was prepared using the FastTrack 2.0 Kit (Invitrogen Corp.) and 1 μ g was used to generate cDNA with the SuperScript Plasmid System Kit (GIBCO BRL). This procedure yielded fragments predominantly containing SalI-MluI linkers ligated to the 5' end and oligo(dT)/dA-NotI primer/adapters at the 3' end of the molecules. NotI-digested cDNA was ligated into the XhoI and NotI sites of pCP507 (35) that had been modified to include a NotI site. The ligation mix was electroporated into DH10B cells (GIBCO BRL) that were plated onto 20 15-cm petri dishes at a density of 25,000 colonies/plate. Colonies were scraped off each plate, and a portion was used for plasmid DNA purification, generating an unamplified library consisting of 20 master pools containing 25,000 clones each or a total of 550,000 clones. The remainder was saved as glycerol stocks in 15% glycerol. Of 20 random clones tested, three did not contain an insert, one contained three inserts, and one appeared to be rearranged. If these clones are excluded, then the average insert size was 1.6 kb.

pCP507 was modified from the mammalian expression vector pCDNA-3 (Invitrogen Corp.) and includes the coding sequence for a GFP variant (S65T, V163A) followed by a linker sequence and an XhoI site without an intervening termination codon. Therefore, expression of the library results in GFP fused to cDNA-derived polypeptide sequences.

Isolation of cDNA Clones by a Visual Screen

For each master pool, 20 starting pools of 2,000 clones each were generated from a glycerol stock by plating the appropriate dilution onto 20 10-cm petri dishes. Colonies were scraped off and glycerol stocks and miniprep DNA (Wizard Plus; Promega) were prepared. BHK cells plated at a density of 16,000 cells/cover slip were transfected (see below) with 1 to 2 μ g of DNA. The next day, cells were permeabilized in 400 μ l PBS⁺ (PBS containing 0.88 mM CaCl₂ and 0.49 mM MgCl₂) with 0.03% Triton X-100 (TX-100) for 5 min, washed briefly with PBS⁺, and fixed with 2% paraformaldehyde in PBS⁺ for 10 min. Fixed cells were visually inspected at a magnification of 63 for distinctive fluorescence patterns (e.g., NE, ER, etc.) resulting from the expressed GFP fusions by scanning a coverslip completely. The pool size of 2,000 clones was chosen to allow for efficient sampling of the library, on the one hand, and to allow for the isolation of candidate clones in a reasonable number of rounds of sib selection (three), on the other. At that pool size, with a typical transfection efficiency of 20–50%, a distinctive fluorescence pattern could be recognized three to seven times on any given coverslip. Starting pools yielding positive clones were subdivided into 20 pools of 200 clones and screened visually. To screen the following subdivision, colonies were first picked into four 96-well microtiter plates and cells from three microtiter plate columns were pooled (24 colonies). Finally, clones from single wells of the positive microtiter pool were analyzed and the identity of the positive clone was ascertained by sequencing the ends of the cDNA insert with vector specific primers (BioPolymers Facility) and by searching the National Center for Biotechnology Information databases using the BLAST algorithm (1).

After cloning five lamin A/C clones, a secondary screen was introduced to eliminate further cloning of lamins A/C. Pools that yielded strong nuclear rim patterns, when expressed, were retransfected into BHK cells that were subsequently stained with mAb 1E4 (28). This antibody recognized human, but not hamster, lamin A/C. Pools positive for antibody staining were not followed.

Plasmid Construction and Manipulation

Cyan and yellow (ECFP and EYFP; ref. 29) versions of visually localized proteins (VLPSs) were constructed by excising the cDNA insert from the library vector with MluI and NotI and subcloning it into pECFP-C3mn or pEYFP-C3mn. These vectors were derived from pECFP-C3 and pEYFP-C3 (provided by J. White [EMBL Heidelberg] based on vector pEGFP-C3 [CLONTECH Laboratories, Inc.]) by destroying the internal MluI site and adding MluI and NotI sites to the polylinker. The modified region of the polylinker reads: aagcttcacgcgtcgaattctgcagtcgacggcggcgcgggtacc.

To construct LBR-S the coding region of the first 238 amino acids of human LBR was amplified by PCR from clone QY-1 (46), which was provided by H. Worman (Columbia University, NY). PCR primers included a Kozak consensus sequence at the 5' end and restriction sites to clone the insert into the XhoI and ApaI sites of pEGFP-N1 (CLONTECH Laboratories, Inc.). The LAP2-S construct included coding sequence of amino acids 237–453 of rat LAP2. This region was PCR amplified from clone 4b (15), which was provided by L. Gerace (Scripps Research Institute, La Jolla, CA). It was inserted into the XhoI and ClaI sites of pCP507. YFP-emerlin was generated by PCR amplification of the full-length coding se-

quence from the GFP-cDNA fusion library and its insertion into the MluI and NotI sites of pEYFP-C3mn.

VLP54 truncations were made by PCR amplifying the regions of interest with MluI and NotI restriction sites at the end and inserting them into pEYFP-C3mn. The 54C construct was made by PCR amplifying the predicted coding region of nurim with BglIII and EcoRI sites at the ends and inserting it into pEGFP-N1 (CLONTECH Laboratories, Inc.). VLP54 point mutants of the yellow version of VLP54 were made with the Gene-Editor kit (Promega Corp.) and deletions were constructed by overlapping PCR. $\Delta 1$ had the sequence SLRPLLGIPESGPGDARQ replaced with GAPGALV and $\Delta 2$ had VYYHVLGLGEPLALKSPRALRFLSHLRHPVC also replaced with GAPGALV.

Cell Culture and Transfections

All cells (BHK-21 [hamster and CCL-10; American Type Culture Collection], Vero [African green monkey and CCL-81; ATCC], HeLa [human and CCL-2; ATCC], and DF1 [chicken]) were grown in DME supplemented with 10% defined FBS (HyClone Laboratories Inc.), 100 U/ml each penicillin and streptomycin, and GlutaMAX-1 (GIBCO BRL) in a humidified 37°C incubator with 5% CO₂. BHK and DF1 cells grown on 18-mm-round coverslips were transfected using the calcium phosphate method (20). They were incubated for ~20 h after transfection before analysis. Vero cells were also grown on 18-mm-round coverslips, but lipofectamine (GIBCO BRL) was used for transfection (1.6 μ l lipofectamine with 0.75 μ g DNA per coverslip). For immunoblotting and stable cell line generation, calcium phosphate transfections of BHK cells were scaled up to 10-cm plates. Stable cell lines were selected with geneticin (GIBCO BRL) and single colonies were generated by limited dilution cloning.

Extractions of Cells Analyzed Visually

Transfected cells on coverslips were washed in PBS⁺, controls were fixed directly in 3% paraformaldehyde in PBS, and permeabilized with 0.5% TX-100 in PBS⁺ for 4 min. The other cells were extracted on ice in 400 μ l PBS⁺ containing 1% TX-100 or PBS⁺ containing 1% TX-100 and 350 mM NaCl for 10 min. After extraction cells were washed twice with PBS⁺, and then fixed with 3% paraformaldehyde. All cells were stained with 0.2 μ g/ml Hoechst dye 33258 (Sigma Chemical Co.). Transfected cells on coverslips were subjected to a nuclear matrix preparation as described (40). In brief, cells were permeabilized with 0.5% TX-100 on ice, extracted with 250 mM ammonium sulfate, and digested with 400 U/ml DNaseI at 32°C for 40 min. Finally, the matrices were fixed and stained with Hoechst as above.

Antinurim Antibody Production

Rabbit polyclonal antibodies were raised against each of two peptide sequences in nurim, 54.1 (KSPRALRFLSHLRHPVC) and 54.2 ([C]QRK-LHLLSRPQDGEAE), located on opposite sides of the last predicted membrane anchor. 54.1 and 54.2 (Biopolymers Laboratory) were coupled via a terminal cysteine to three different carrier proteins keyhole limpet hemocyanin (Calbiochem-Novabiochem Corp.), BSA (Calbiochem-Novabiochem Corp.), and ovalbumin (Pierce Chemical Co.), and rabbits were immunized by successive injections with each peptide conjugate (Cocalico Biologicals, Inc.). Two rabbits were used for each peptide yielding antisera 251 and 252 against peptide 54.1 and antisera 253 and 254 against peptide 54.2. Each antiserum was affinity-purified over Sulfo-Link (Pierce Chemical Co.) columns to which either peptides 54.1 or 54.2 had been coupled, and then concentrated over a hydroxyapatite (Bio-Rad Laboratories) column.

Analysis of GFP-fusions and Endogenous Nurim by Immunoblotting

Cells were removed from 10-cm tissue culture plates with PBS plus 5 mM EDTA, pelleted at 1,000 *g* for 5 min and washed in cold PBS⁺. For some experiments (see Fig. 6 a), the cells were divided directly into three samples, pelleted, and treated with one of three extraction conditions: PBS⁺ alone (control), PBS⁺ with 1% TX-100, or PBS⁺ with 1% TX and 350 mM NaCl, each supplemented with 1 mM DTT and protease inhibitor cocktail. Samples were incubated for 30 min on ice. The insoluble material was pelleted at 3,500 *g* for 10 min, washed with PBS⁺, and repelleted. In other experiments (see Figs. 4 b and 6 b) total membrane fractions (BHK cells and BHK cells overexpressing nurim coding sequence) or nuclei (HeLa cells and Vero cells) were first isolated by hypotonic lysis. Cells were incubated in 10 vol of cold hypotonic lysis buffer (HLB: 10 mM Tris-Cl, pH 7.5, 10

mM NaCl, 1.5 mM MgCl₂, 1 mM DTT, and protease inhibitor cocktail) until swollen and lysed by passage through a ball bearing homogenizer. Extent of lysis was monitored by phase-contrast microscopy. Total membranes were collected by centrifugation at 25,000 *g* for 10 min, washed in cold isotonic buffer (ILB: 10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM DTT, and protease inhibitor cocktail), and resuspended in nuclei resuspension buffer (NRB: 15 mM Hepes, pH 7.4, 80 mM KCl, 15 mM NaCl, 250 mM sucrose, 1.5 mM MgCl₂, 1 mM DTT, and protease inhibitor cocktail). Nuclei were collected by centrifugation at 3,500 *g* for 5 min, washed, and repelleted twice in NRB. Aliquots from these samples were either directly solubilized in SDS-PAGE sample buffer (see Fig. 4 b) or extracted as described above (see Fig. 6 b). To analyze supernatants, proteins were precipitated with TCA (15% final), pelleted at 25,000 *g* for 10 min, and pellets were washed twice with acetone. Final pellets were solubilized in SDS-PAGE sample buffer at 65°C for at least 30 min. For immunoblot analysis, proteins were separated by SDS-PAGE and transferred to nitrocellulose. The blots were probed either with rabbit polyclonal anti-GFP antibodies (provided by P. Silver, Dana-Farber Cancer Institute, Boston, MA) at a dilution of 1:3,000 or with rabbit polyclonal antinurim antibodies at a dilution of 1:4,000 in TBS/0.1% Tween 20 with 5% dry milk and proteins were detected by chemiluminescence (Renaissance; NEN Life Science Products).

Immunofluorescence Staining

Cells on 18-mm-round coverslips were washed with PBS; for antinurim antibodies cells were fixed with -20°C methanol for 4 min and rehydrated in PBS; and for mAb 414 (BABCo) cells were fixed with 3% paraformaldehyde in PBS with subsequent permeabilization with 0.5% TX-100 for 4 min. Fixed cells were blocked in PBS with 10 mM glycine, 2 mM Na₂S₂O₈, and 10% FBS (block) for 30 min. Primary antibody incubations were done for 45 min in block containing affinity-purified antinurim antibodies diluted 1:1,500 or mAb 414 diluted 1:5,000. Cells were washed with PBS, blocked for >30 min, and incubated for 30 min with rhodamine anti-rabbit antibodies for nurim staining or rhodamine anti-mouse for mAb 414 diluted 1:400 in block. Final washes were performed in PBS⁺.

Fluorescence Microscopy

Cells were mounted in 90% glycerol/10% 0.2 M Tris, pH 7.4. For all experiments, except those shown in Figs. 7, 8 b, and 10, cells were viewed on an Axioplan II microscope (Carl Zeiss), equipped with an Orca 12-bit-cooled CCD camera (Hamamatsu Photonics). Images were captured and scaled using Image-Pro Plus 3.0 software (Media Cybernetics) with additions by Phase 3 Imaging Systems. A Zeiss 63 \times plan Apochromat oil immersion objective was used. For experiments in Figs. 7 and 8 b, a DeltaVision microscope system (Applied Precision Instruments) built around a Zeiss Axiovert microscope and with a PXL CCD camera (Photometrics Ltd.) was used with a Zeiss 100 \times plan Apochromat oil immersion objective (see Fig. 7) or a 63 \times plan Apochromat oil immersion objective (see Fig. 8 b). Filters for visualization of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) were from Chroma Technology Corp. After acquisition of images with DeltaVision software they were exported to either NIH Image 1.62 (National Institutes of Health) for Fig. 7 or ImagePro Plus for Fig. 8 b, and scaling and overlaying were performed in these applications. Final preparation of all figures was done using Canvas 5.0 (Deneba Systems, Inc.).

Fluorescence Recovery after Photobleaching (FRAP)

FRAP experiments were performed with a Zeiss LSM 410 using the 488-nm line of a 100-mW Kr laser and a Zeiss 100 \times Plan Apochromat oil immersion objective. Transiently transfected BHK cells were observed at room temperature and imaged at 3% transmission. Cells were bleached for 30 s at 100% transmission and observed every 11 s for 20 images, and then every minute for 5 images.

Results

A Visual Screen for Identifying Localized Proteins

To identify proteins localized to different subcellular compartments, a library was constructed with poly(A)-primed cDNA prepared from a human osteosarcoma cell line. The

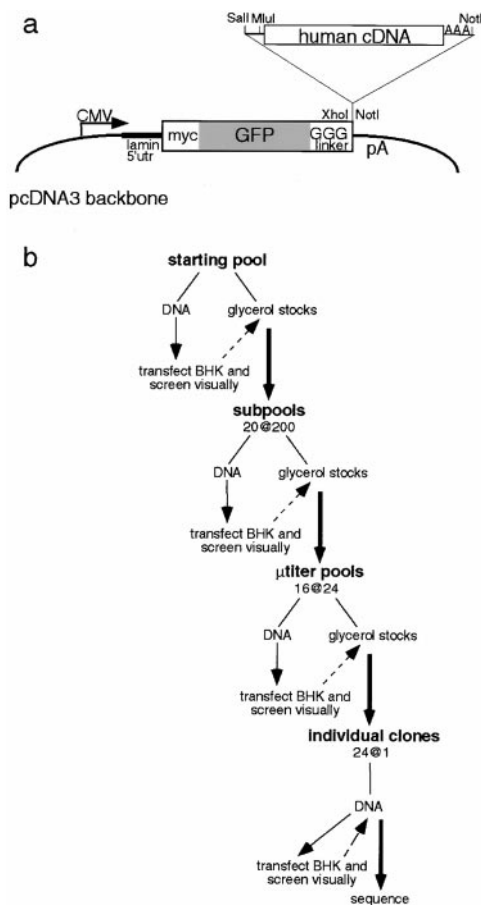


Figure 1. Overview of the visual screen. (a) Construction of the expression library. Human cDNAs from an osteosarcoma cell line were inserted into a mammalian expression vector derived from pcDNA3. The vector was modified by the addition of part of the 5' untranslated region of lamin A (lamin 5' utr), as well as the coding sequences of a myc tag, a GFP variant (S65T and V163A), and a flexible linker (GGGLDPRVR). The library was initially generated as 20 master pools of 25,000 clones each. (b) Screening the expression library. Starting pools were generated by subdividing each master pool into 20 pools of 2,000 clones. These pools were screened for localized proteins by transfecting each pool into $\sim 2 \times 10^4$ cells grown on a coverslip. The transfection efficiency varied from 20–50% meaning that each clone in the pool was expressed in several cells. Transfected pools were screened visually by epifluorescence microscopy. Pools that gave rise to interesting patterns were retrieved from glycerol stocks and further subdivided and screened until single clones were isolated.

library vector allowed expression of cDNAs in tissue culture cells as COOH-terminal polypeptide fusions to GFP (Fig. 1 a). While GFP is larger than most antibody epitope tags, it forms a tight structure and has been fused to many proteins without disrupting them. Its advantage for a large-scale screen is that it can be directly visualized in transfected cells without the manipulations required for epitope tags.

To screen the expression library, we transfected BHK cells with pools of clones generated by subdividing the library and examined the cells for distinct patterns of GFP fluorescence (Fig. 1 b). Cells expressing GFP fusion pro-

teins were extracted with detergent to remove soluble GFP fusions, including many derived from expression of out of frame cDNAs. Extracted cells were fixed to preserve them for analysis. When a desired pattern was identified, sib selection, the repeated subdivision and rescreening of pools was used to isolate the clone responsible for the distinct pattern. Three rounds of subdivision were required to generate single clones. Using this method, it was often possible to identify a specific pattern in a pool of 2,000 clones and to isolate the clone responsible for that pattern.

Validation of the Visual Screen

A visual screen had not been performed previously in mammalian cells, so we determined its effectiveness before assigning significance to our results. We isolated clones that exhibited a variety of patterns and determined whether they contained coding sequences of proteins normally targeted to the structure in which GFP fluorescence was observed. We screened a total of 220 starting pools, and identified 32 that contained patterns we wished to investigate further. Many of the positive pools produced several different patterns in transfected cells and, in fact, yielded multiple localized clones. In all, we isolated 60 independent clones with interesting cellular localizations, which can be divided into two groups. One group of clones encoded fusion proteins that were not clearly localized to a single compartment or were localized to a compartment that was not readily identifiable. The other group, 27 clones, expressed fusion proteins that were clearly targeted to single defined compartments, for example the NE, in all transfected cells.

The group of 27 clones targeted to single, defined subcellular compartments included 25 sequences of known proteins (Table I). The fluorescence patterns of most of these fusion proteins were consistent with their localization to the same compartment as the endogenous protein (exceptions noted in Table I). In most cases the clones encoding correctly localized fusions contained the entire coding sequence of the endogenous proteins. Examples of the patterns obtained with isolated clones are shown in Fig. 2. The correct localization of GFP fusions to known proteins confirmed that this visual screen yields meaningful results when a pattern of interest can be clearly identified.

Two of the clearly localized clones encoded novel proteins. One of the fusion proteins, VLP27, localized to interphase microtubules. The other fusion protein, VLP54, was targeted to the nuclear envelope. Because most of the clearly targeted fusions to known proteins were localized like their endogenous counterparts, it is likely that VLP27 and VLP54 also are localized like the cellular proteins they represent.

In the screen, we also followed patterns that we could not identify as corresponding to a particular subcellular compartment and this led to the identification of a group of clones whose expression pattern was not easily classified. This group contained fusions localized to several compartments; for example, multiple organelles in the secretory pathway. It also contained fusions that were localized inconsistently, with a large proportion of the fusion

Table I. VLP Clones Encoding Clones Localized to a Single Distinct Compartment

Localization	VLP	Coding sequence (cds)	5' end of cds
ER	6	Heme oxygenase-2	Included
	8	Phosphatidyl inositol synthase	Included
	16	Signal peptidase 25-kD subunit	Included
	25	Sec61 β	Included
	47	Signal peptidase 120-kD subunit	Included
	59	Vti2*	Included
Cytoskeleton	11	β -Actin	Included
	50	Vimentin	Included
	61	Neutral calponin	Included
Mitochondria	32	ADP/ATP translocase	Included
Chromatin	29	HMG-17	Included
	51	Histone H1	Included
Nucleoli	65	Ribosomal protein L27*	Missing (-3aa)
Centrosomes	31	CAD: ATCase domain in clone*	‡
Nuclear lamina	4	Lamin A	Included
	5	Lamin C	Included
	7	Lamin A or C	Included
	12	Lamin C	Included
	13	Lamin A	Included
	28	Lamin B	Missing (-87aa)
Nuclear pores	§	p62	Missing (-221aa)
	23	SUMO-1	Included
	35	RanGAP1	Missing (-397aa)
	55	Ran	Included
	56	SUMO-1	Included

*Most clearly localized fusions were targeted to the same organelle as the endogenous proteins they represent. There are three possible exceptions. The aspartate transcarbamylase (ATCase) domain of CAD is unlikely to be targeted to centrosomes (8). In other applications, for example immunofluorescence and biochemical fractionation, centrosomes are notoriously sticky, so this may explain why we found a clone that does not encode a known centrosomal component localized there. Vti2 may also be an exception as the endogenous protein has not been localized. Ribosomal protein L27 is correctly targeted to nucleoli, but we do not know whether it is incorporated into ribosomes.

‡CAD is a multifunctional protein involved in de novo pyrimidine synthesis that encodes carbamyl-phosphate synthetase, dihydroorotase, and ATCase activities. This clone includes the complete coding region for the ATCase domain, which is encoded at the 3' end of the transcript, and the upstream linker.

§This clone was isolated in a test of the screen before we initiated a numbering system and will be referred to as p62-GFP.

spread diffusely through the cytoplasm, and a variable amount on a specific structure. The sequence of these clones in some cases made the pattern understandable; for example, many of the clones that were localized to several secretory organelles encoded short hydrophobic sequences or fragments of membrane proteins. In other cases, the clones encoded fragments of proteins, unidentifiable sequence, or poorly characterized proteins. Whereas some of the fusions in this class are likely to be interesting, it is not clear that their localization always represents that of an endogenous protein.

Identification of Known Nuclear Envelope Proteins in the Visual Screen

The NE was a pattern that could be clearly identified in the visual screen and we cloned a number of GFP fusions to known NE proteins. The most abundant class of NE proteins found was lamins. This is likely to reflect their abundance within the cell and the ease with which tagged lamins can be incorporated into the lamina. GFP-lamin fusions gave very clear, bright fluorescence at the nuclear

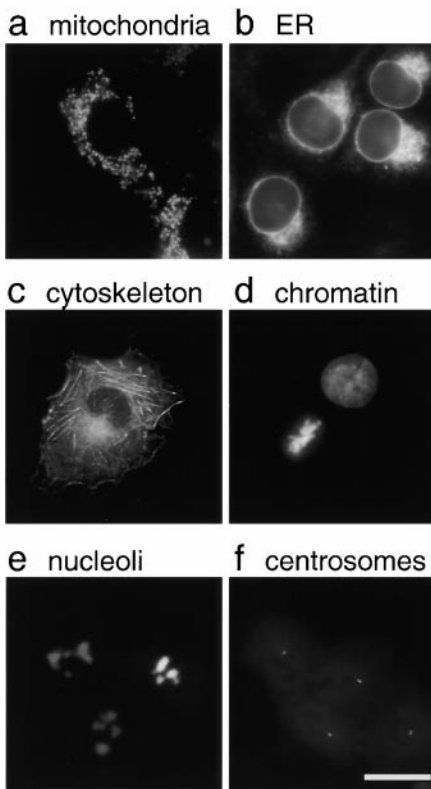


Figure 2. Examples of fluorescence patterns generated by expression of individual clones isolated in the visual screen. Individual clones were transiently transfected into BHK cells. (a) A mitochondrial pattern is shown by VLP32, a GFP fusion to ADP/ATP translocase; (b) an ER pattern by VLP16, a fusion to the signal peptidase 25 kD subunit; (c) a cytoskeletal pattern by VLP11, a fusion to β -actin; (d) a chromatin pattern by VLP51, a fusion to histone H1; (e) a nucleolar pattern by VLP65, a fusion to ribosomal protein L27; and (f) a centrosomal pattern by VLP31, a fusion to the ATCase domain of CAD. Bar, 20 μ m.

rim and some internal nuclear structures (Fig. 3 a) that are likely to be invaginations of the NE (14). After identifying five independent clones of lamin A or C (which differ only in a splice variation at their COOH terminus) in the first 100 pools screened, we introduced a secondary screen to eliminate pools that exhibited NE fluorescence and expressed human lamin A or C. After this modification, we cloned only lamin B.

We also identified several GFP fusions to proteins localized to nuclear pores. These fusions were distinguishable from fusions to lamins because they appeared punctate at the nuclear periphery (Fig. 3 b). Of these clones, two encoded SUMO-1, a ubiquitin-related protein that modifies, among other proteins, RanGAP1 and targets it to RanBP2 at the nuclear pore (24, 27). We also identified a fusion to the COOH-terminal portion of RanGAP1, which is the region of the protein required for modification by SUMO-1 and targeting to the nuclear pore (27), and a fusion to Ran. Although Ran is present both inside and outside the nucleus, our ability to detect it at the pores likely reflects its shuttling between the nucleus and cytoplasm. We also found one fusion to a core component of the nuclear pore complex, p62. The identification of clones encoding lamins

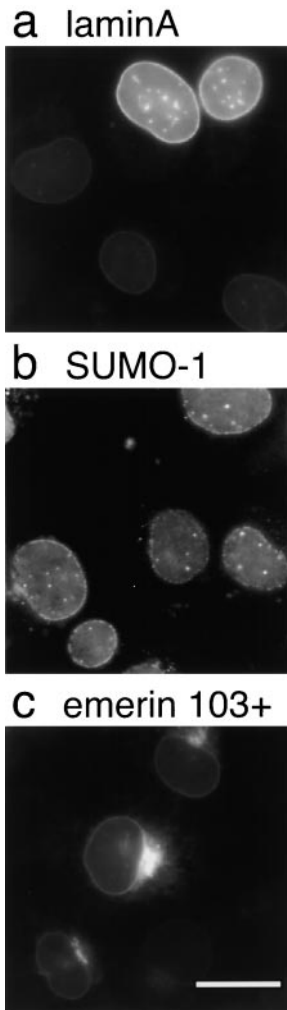


Figure 3. Examples of fluorescence patterns generated by GFP fusions to known NE proteins. BHK cells were transiently transfected with isolated clones (a) VLP4, a fusion to lamin A; (b) VLP23, a fusion to SUMO-1; and (c) VLP33, a fusion to the COOH-terminal half of emerin (starting at amino acid 103). Bar, 20 μ m.

and nuclear pore-associated proteins confirmed that we could recognize and follow NE components through the screen.

We only isolated one fusion to a known membrane protein of the NE, emerin, and this clone (VLP33) proved not to be specifically targeted to the nuclear envelope (Fig. 3 c). The fusion protein was also present in the peripheral ER and structures next to the nucleus that may be the Golgi complex. VLP33 contains amino acids 103–254 of emerin and is not expected to be targeted to the NE because a deletion of amino acids 95–99 has been shown to partially disrupt NE localization of emerin (12).

A Novel Multispanning Membrane Protein Tightly Associated with the Nucleus

The clone we isolated that encoded a membrane protein targeted to the NE, VLP54, did not contain sequences related to known NE membrane proteins. The only similar protein found in a BLAST search was a hypothetical protein from *Mycobacterium tuberculosis* that was 29% identical to a 139–amino acid stretch of VLP54, but there were a number of human and rodent expressed sequence tags (ESTs) that aligned perfectly, or very closely, with regions of the nucleotide sequence of VLP54. No possible transla-

tion initiation ATG was present near the beginning of the sequence included in VLP54, but several of the ESTs extended slightly further in the 5' direction and comparisons suggested that the first nucleotide present in our clone was the G from an ATG codon. One EST also indicated that a good consensus translation initiation sequence (22) preceded the ATG. The sequence information suggests the endogenous protein contains 262 amino acids and has a molecular weight of 29 kD. The protein is predicted to contain five transmembrane domains with short intervening loops (Fig. 4 a). We named the protein nurim (for nuclear rim protein).

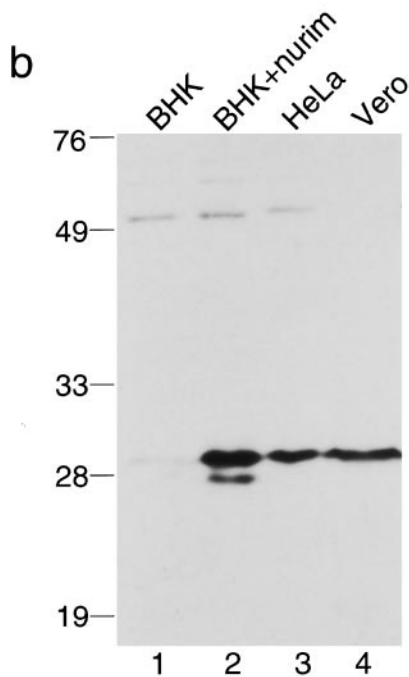
To characterize the endogenous protein we made polyclonal antibodies, two against each of two peptides in nurim (see Fig. 4 a). All four affinity-purified antibodies recognized a protein of \sim 30 kD in immunoblots of nuclear extracts from human, monkey (Fig. 4 b, lanes 3 and 4), and rat liver (not shown) cells, but not of extracts from BHK cells (Fig. 4 b, lane 1). Further evidence that VLP54 contained the full-length coding sequence of nurim was derived by comparing the size of an untagged version of VLP54 expressed in BHK cells with that of endogenous nurim in HeLa and Vero cell nuclei. The protein in transfected cells had the same size as the endogenous protein (Fig. 4 b, lane 2 versus lanes 3 and 4), although a slightly smaller band, which may be a degradation product, was also present in transfected cells.

At low expression levels in transiently transfected cells, the predominant GFP pattern of VLP54 was nuclear rim. This pattern was also seen in a stable cell line we constructed (Fig. 4 c). At higher expression levels in transiently transfected cells, VLP54 was also present in the peripheral ER (see Fig. 5), suggesting that its targeting to the NE may be easily saturable. To determine whether endogenous nurim is also localized to the NE, we used the affinity-purified peptide antibodies for immunofluorescence. All four antibodies stained the NE but not peripheral ER (Fig. 4 d and not shown), confirming that endogenous nurim is localized like VLP54 to the NE.

The known membrane proteins targeted to the NE, both nuclear pore components and nonpore proteins, are resistant to extraction with 1% TX-100 (2, 12, 13, 19, 37). Therefore, we tested whether VLP54 shares this characteristic. When LBR-S, a fusion of the nucleoplasmic and first transmembrane domains of LBR to GFP, was transfected into cells, it remained at the nuclear periphery and also inside the nucleus after extraction with 1% TX-100 (Fig. 5). LBR-S may be present within the nucleus as well as at its periphery because it contains chromatin- as well as lamin-binding domains. In the same assay, a GFP-LAP2 fusion (LAP2-S) that contained determinants for binding lamins, but not chromatin, remained only at the nuclear periphery after extraction with 1% TX-100 (not shown).

Like LBR-S and LAP2-S, VLP54 was still present at the nuclear rim after extraction with 1% TX-100. Interestingly, after extraction with 1% TX-100 and high salt (\sim 500 mM) VLP54 remained at the nuclear rim, whereas LBR-S and LAP2-S were removed by this condition (Fig. 5 and not shown). VLP54 also remained associated with the nuclear periphery after a series of extractions that left only the nuclear matrix (Fig. 5). Thus, VLP54 is very tightly associated with the edge of the nucleus and can be consid-

a MAPALLLIPAAALASFILAFGTGVEFVRFTS 30
 LRPLLGGIPESGGPDARQGWLAALQDRSIL 60
 APLAWDLGLLLLFGVQHSLMAAERVKAWTS 90
 RYFGVLQRSLYVACTALALQLVMRYWEPIP 120
 KGPVLWEARAEPWATWVPLLCFVLHVISWL 150
 LIFSILLVFDYAELMGLKQVYYHVLGLGEP 180
 LALKSPRALRLFSHLRHPVCVELLTVLWVV 210
 PTLGTDRLLLAFLLTLYLGLAHGLDQQDLR 240
 YLRAQLQRKLHLLSRPODGEAE 262



c VLP54 stable cells **d** endogenous nurim

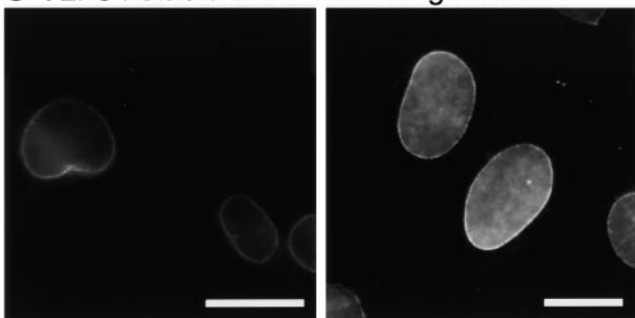


Figure 4. Nurim, a new NE membrane protein isolated in the visual screen. (a) Predicted amino acid sequence of nurim based on the coding sequence included in VLP54. Predicted transmembrane domains are shaded gray and peptide sequences 54.1 and 54.2 used to generate antibodies are underlined. These sequence data are available from GenBank/EMBL/DDBJ under accession number HSNRM29 AF143676. (b) An immunoblot with affinity-purified antibody 254 to peptide 54.2. Lane 1 was loaded with protein from 8×10^5 BHK cells; lane 2 with 1×10^5 BHK cells transiently transfected with an untagged version of VLP54; lane 3 with 8×10^5 HeLa nuclei; and lane 4 with 8×10^5 Vero nuclei.

ered a nuclear matrix constituent. Control experiments demonstrated that VLP6, a GFP fusion to the ER protein heme oxygenase-2 (HO-2), was readily extracted with 1% TX-100 (Fig. 5). Similar results were obtained with GFP fusions to two other ER proteins, VLP25 (Sec61 β) and VLP8 (phosphatidylinositol synthase), the latter of which, like VLP54, has multiple transmembrane domains (not shown). VLP54 present in the peripheral ER in highly expressing cells was also readily extracted by 1% TX-100 (Fig. 5), indicating that nuclear rim localization is required for it to become detergent-inextractable.

To confirm the salt- and detergent-resistant association of VLP54 with the nucleus, we extracted a stable cell line expressing VLP54 and a stable cell line expressing two ER proteins with 1% TX-100 or 1% TX-100 and high salt and analyzed the nuclear pellet by immunoblotting with GFP antibodies. A large proportion of VLP54 remained with the nuclear pellet, whereas the two ER proteins were released from the pellet (Fig. 6 a).

The tight association of VLP54 with the nucleus was not limited to the GFP fusion protein. When Vero cells were extracted with 1% TX-100 and 1% TX-100 plus high salt as in Fig. 5 and analyzed by immunofluorescence with nurim antibodies, bright nuclear rim staining was present after both extractions (not shown). Immunoblot analysis confirmed that endogenous nurim in HeLa nuclei remained largely in the nuclear pellet after extraction with 1% TX-100 or 1% TX-100 plus salt (Fig. 6 b). Similar results were obtained with nuclei from Vero cells (not shown). Taken together, these data show that both endogenous nurim and its GFP fusion are targeted and tightly bound to the nuclear periphery. Because VLP54 and endogenous nurim behaved identically, we used VLP54 in additional experiments and will refer to it as GFP-nurim.

Nurim Is Not Localized to Nuclear Pores

To test whether nurim is a membrane protein of the nuclear pore, we compared the distributions of GFP-nurim and GFP-p62, a GFP fusion to the COOH-terminal half of nucleoporin p62, with that of nuclear pores. Nuclear pores were localized with mAb 414, a well-characterized mAb that recognizes several nuclear pore proteins (9, 10). GFP-p62 gave a punctate staining pattern (Fig. 7 d) similar to that seen with mAb 414 (Fig. 7 e). Since the intensity of nuclear pore labeling with the antibody and GFP fusion was often different, the color overlay is not uniformly yellow (Fig. 7 f). However, a magnified view shows that the pattern of GFP-p62 and mAb 414 dots was largely overlapping, with many dots labeled by both probes (Fig. 7, j-l). The distribution of GFP-nurim at the nuclear surface was also slightly punctate, but the dots were less pronounced than those seen with GFP-p62 (Fig. 7, versus d). No relationship between the pattern of GFP-nurim and mAb 414 was apparent (Fig. 7, a-c and g-i). Therefore, we

The position of molecular mass markers is shown at the left and their size is in kD. (c) Nuclear rim fluorescence is shown with a BHK stable cell line expressing VLP54 and (d) Vero cells stained with affinity-purified antibody 253 to peptide 54.2. Bars, 20 μ m.

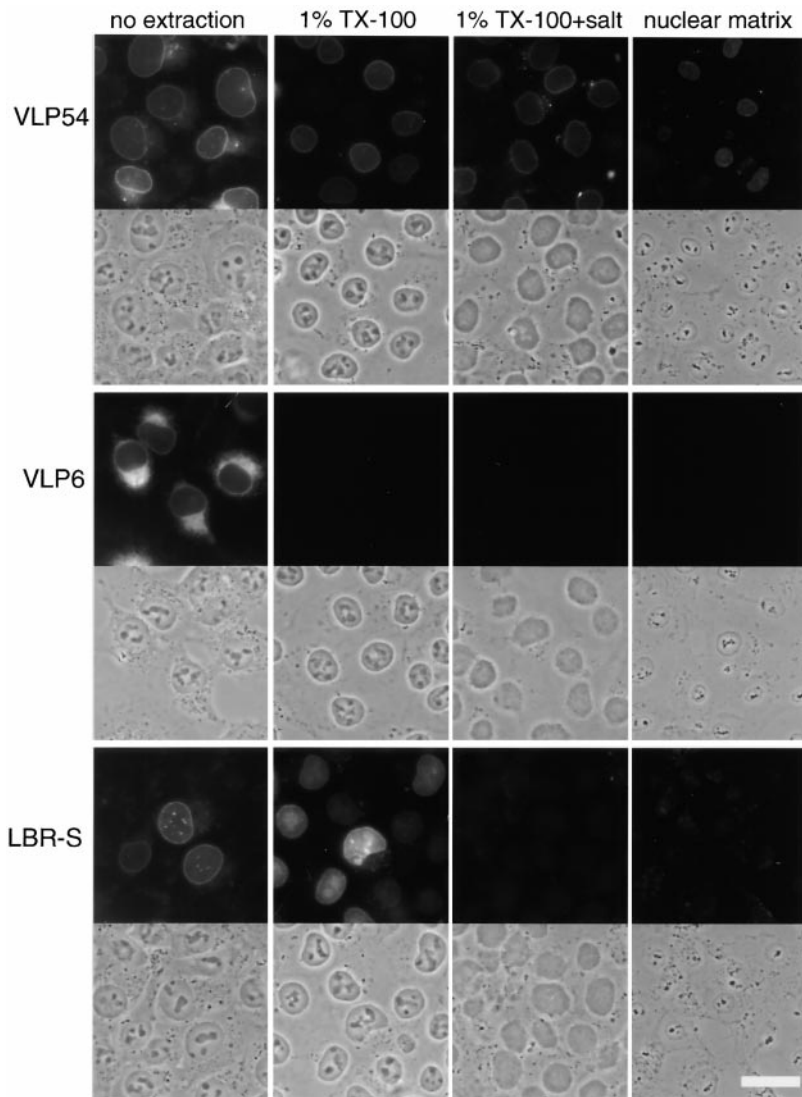


Figure 5. Extractions of cells expressing VLP54, VLP6 (a GFP fusion to the ER protein HO-2), and LBR-S (a GFP fusion to a truncated LBR). BHK cells were transiently transfected with expression constructs and fixed (no extraction) or extracted on ice with 1% TX-100 in PBS (1% TX-100), or 1% TX-100 in PBS supplemented with 350 mM NaCl (1% TX-100 + salt), or subjected to a nuclear matrix preparation (nuclear matrix) before fixation. Upper panels show GFP fluorescence and lower panels show corresponding phase-contrast images. All GFP images were taken at the same exposure and subsequently scaled identically. Bar, 20 μ m.

conclude that nurim is targeted to the NE without being localized to nuclear pores.

Nurim Is Targeted to the NE by a Less Conserved Mechanism than the Lamin-associated Class of Proteins

To compare nurim to the lamin-associated proteins we determined how these proteins are targeted in nonmammalian cells. We reasoned that the lamina is a structural feature of vertebrate cells that should be well-conserved and so mammalian proteins that bind to the lamins should be targeted to the NE in nonmammalian vertebrate cells. When we expressed the GFP fusion proteins LBR-S and LAP2-S in chicken fibroblasts, both were targeted to the NE and remained at the nucleus after extraction with 1% TX-100 as they had in mammalian cells (Fig. 8 a). Both wild-type proteins contain chromatin- and lamin-binding domains, but the LAP2-S construct does not contain the region to which chromatin-binding has been mapped (16). Thus, it is most likely that the lamin-binding domain is functioning to target LAP2-S in the chicken cells. For

comparison, we transfected chicken cells with a GFP fusion of an ER protein, VLP25 (Sec61 β). As in mammalian cells it gave a reticular ER pattern and was extracted by 1% TX-100 (Fig. 8 a). The pattern of GFP-nurim was indistinguishable from that of VLP25 and GFP-nurim was also extracted by 1% TX-100 (Fig. 8 a). The mechanism of nurim targeting to the NE, thus, appears less conserved between species than that of lamin-associated NE membrane proteins.

To test directly whether NE targeting of nurim involved binding to lamin A or C, we cotransfected chicken cells with CFP fusions to lamins, CFP-lamin A (derived from VLP4) or CFP-lamin C (derived from VLP5), and YFP-nurim. These two GFP variants were imaged independently in the same cell using specific excitation and emission filters. Resistance to detergent extraction indicated that both human lamins were incorporated into the lamina of chicken cells (Fig. 8 b and not shown), although lamin C was also present in the interior of the nucleus of unextracted cells (not shown). The distribution of YFP-nurim in cells transfected with CFP-lamins appeared similar to that of GFP-nurim in chicken cells without human lamins

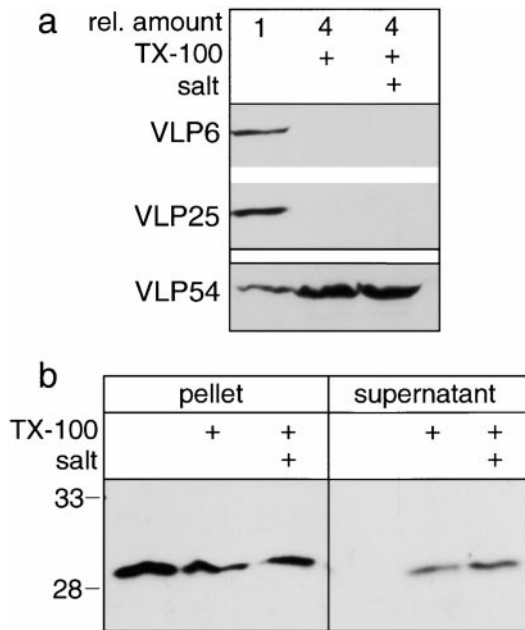


Figure 6. Association of VLP54 and endogenous nurim with extracted nuclear pellets. (a) BHK cells stably expressing either VLP54 or a cyan version of VLP6 (HO-2) and yellow version of VLP25 (Sec61 β) were treated with PBS, PBS with 1% TX-100, or PBS with 1% TX-100 and high salt. Nuclei were pelleted, washed, and analyzed by SDS-PAGE followed by immunoblotting with an anti-GFP antibody. Four times the amount of extracted nuclei compared with unextracted nuclei (1×10^6) were analyzed. (b) HeLa cell nuclei were treated as in a, and analyzed by immunoblotting with affinity-purified antinurim antibody 254. In this experiment supernatants were also collected and the amount from equivalent numbers of cells (3×10^5) were loaded in all lanes.

(Fig. 8 b compared to Fig. 8 a). Also, in detergent-extracted cells containing CFP-lamins, we were never able to detect significant YFP-nurim at the NE (Fig. 8 b, and not shown). Expression of human lamins A and C, thus, did not make chicken cells competent for NE targeting of nurim.

Nurim Does Not Contain an Independent Nucleoplasmic NE-targeting Domain

To determine whether a nucleoplasmic region of nurim could function independently to target the protein to the NE, we examined the targeting of GFP-nurim mutants. Unlike the known members of the lamin-associated class, nurim does not have a long NH₂-terminal extension before its first transmembrane domain that could contain an NE targeting domain. However, it does have several short regions that could extend into the nucleus. We made deletions in these regions: the two longest loops between transmembrane domains, deletions $\Delta 1$ and $\Delta 2$, and the tail after the last transmembrane domain. Removal of the last 16 amino acids of nurim, mutation T16, had no effect on targeting to the NE or detergent inextractability (Fig. 9). On the other hand, mutants $\Delta 1$ and $\Delta 2$ were no longer concentrated in the NE, but were distributed throughout the ER,

and were completely extracted by 1% TX-100 (Fig. 9). The two loops in which we made deletions are predicted to be on opposite sides of the membrane. Obtaining similar results with both deletions does not support the idea that one domain would reach into the nucleus and anchor the protein in the NE.

Because a large proportion of nurim is predicted to consist of transmembrane domains, we also considered the possibility that these regions might be important for NE localization. NH₂ or COOH terminally truncated versions of GFP-nurim containing two, three, or four transmembrane domains were either localized predominantly to the peripheral ER or unstable (not shown). Therefore, we made more targeted changes in the transmembrane domains. We changed each of the three charged residues in the transmembrane domains independently to leucine. One of these mutations, D66L, eliminated targeting to the NE and detergent inextractability (Fig. 9). The other two mutations, R98L and R217L, had an intermediate phenotype with more of the fusion protein present in the peripheral ER and a greater sensitivity to detergent than wild-type GFP-nurim. The disruption of NE localization by point mutations in the transmembrane domains is inconsistent with the idea that nurim contains an independent nucleoplasmic domain with NE targeting determinants. Together with the results from the loop deletion mutants, the behavior of the point mutants suggests either that nurim acts as a very integrated structure, or that multiple regions, including the transmembrane domains, contain determinants for NE targeting.

GFP-nurim Is Anchored at the NE, but the Point Mutant D66L Is Freely Diffusible

To confirm that GFP-nurim is targeted to and tightly bound at the NE, whereas a mutant that is not properly localized diffuses more freely, we performed FRAP experiments. The behavior of fusions of emerin and the NH₂ terminus of LBR to GFP has been examined previously by FRAP. After bleaching areas of the NE or of the peripheral ER, which also contains the fusion proteins when highly expressed, fluorescence returned to the NE more slowly than to the ER (11, 30). Thus, these fusion proteins have a restricted diffusional mobility in the NE.

When we bleached part of the NE of a cell expressing low levels of GFP-nurim, we observed only limited recovery over a 9-min observation time (Fig. 10 a). On the other hand, the NE of cells expressing mutant D66L regained fluorescence during this period (Fig. 10 a). For comparison, we monitored the behavior of VLP25 (a GFP fusion to an ER protein), YFP-emerin, LAP2-S, and LBR-S, and quantitated the percent fluorescence recovery to the NE during the observation period. Like the fluorescence of D66L, that of ER protein VLP25 recovered rapidly to the bleached area (Fig. 10 b). On the other hand, fluorescence of the NE proteins recovered slowly with kinetics similar to those observed for GFP-nurim (Fig. 10 b). This result corroborated the tight association of nurim with components of the nucleus, indicated by its inextractability from the nuclear periphery with detergent and high salt. It also confirmed that mutation of a charged residue predicted to be in the second transmembrane domain disrupts target-

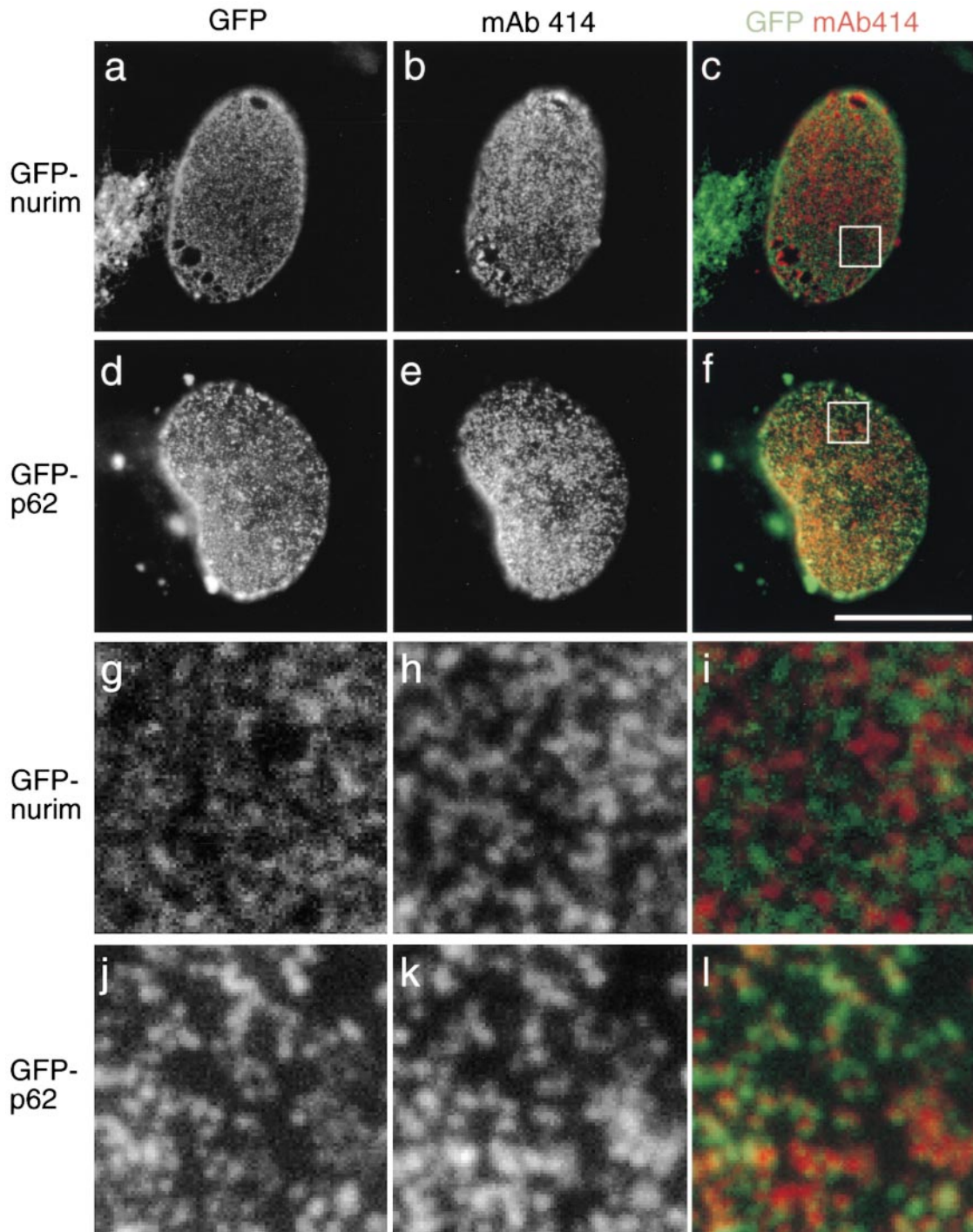


Figure 7. Localization of GFP-p62 and GFP-nurim compared with that of nuclear pores. Vero cells were transiently transfected with either GFP-nurim (a–c and g–i) or GFP-p62 (d–f and j–l). The bottom surfaces of nuclei are shown with enlargements of the regions indicated in c and f shown in g–i and j–l, respectively. (a, d, g, and j) GFP fluorescence and (b, e, h, and k) nuclear pore labeling by mAb 414 followed by a rhodamine secondary antibody. (c, f, i, and l) An overlay with GFP in green and rhodamine in red. Bar, 20 μm .

ing of GFP-nurim to the NE and results in a protein that behaves like a freely diffusible ER component.

Discussion

We have developed a visual screen to identify proteins lo-

calized to specific compartments, such as the NE, in mammalian cells. Precise localization of fusion proteins in whole cells is expected to reflect specific targeting of the endogenous protein and is stringent since it requires the targeting determinants to function in the context of their natural, complex environment. The visual screen obviates the need to make assumptions about how proteins are lo-

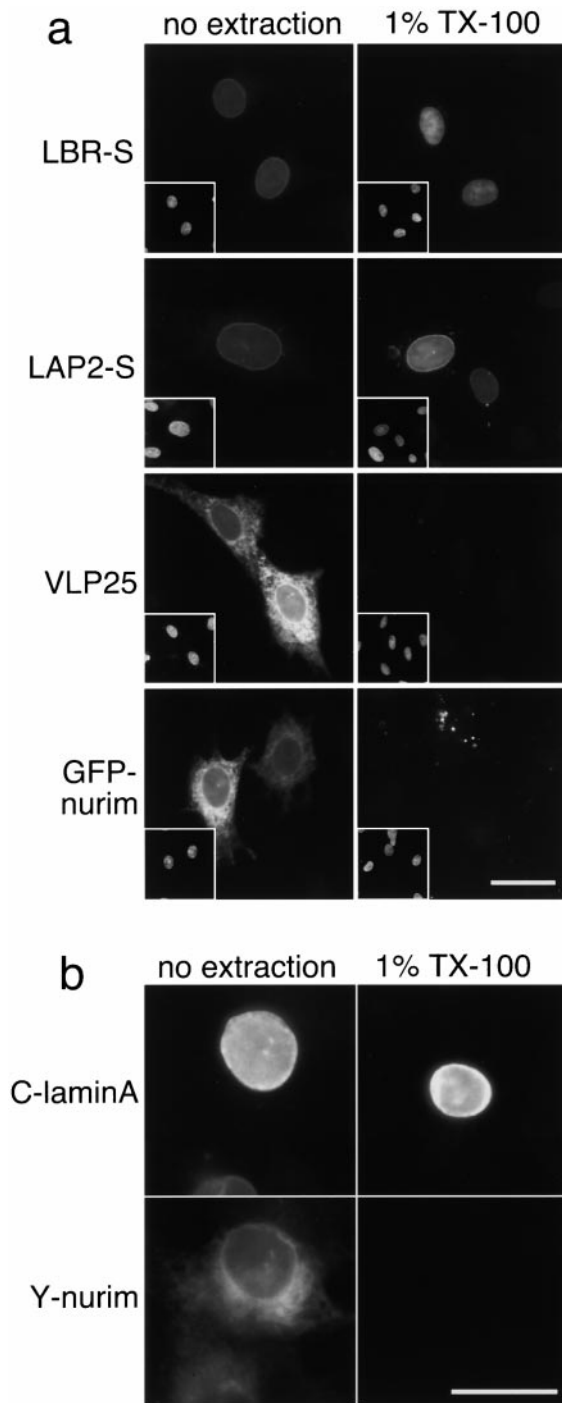


Figure 8. Localization and detergent sensitivity of GFP-fusions in chicken cells. (a) Chicken fibroblasts were transiently transfected with LBR-S and LAP2-S (GFP fusions to NE proteins), VLP25 (a GFP fusion to the ER protein Sec61 β), or GFP-nurim. Cells were either fixed immediately (no extraction) or extracted with 1% TX-100 before fixation (1% TX-100). GFP fluorescence is shown in large images and Hoechst staining in insets. GFP images were taken at the same exposure and scaled identically. (b) Chicken cells were transiently transfected with both CFP-lamin A and YFP-nurim and treated as in a. The cyan and yellow images of the same cells are shown in the top and bottom frames, respectively. All images were taken at the same exposure and subsequently scaled identically. Bars, 20 μ m.

calized to a particular structure and does not require the physical separation of structures from one another.

Although visual screens have been performed in yeast (7, 32), the complexity of the mammalian genome made it impractical for us to screen individual clones as had been done in those cases. Therefore, we used a small pool approach and sib selection to isolate localized clones (23). Small pool sizes increase the likelihood of scoring positive clones because each clone contributes a higher proportion of the signal. The functional pool size in our screen was much smaller than the 2,000 clone starting pool size; it was the number of plasmids expressed in any given cell (perhaps 5–20 with the transfection method used; Lanini, L., and F. McKeon, unpublished results) since each cell was transfected and screened independently.

Expressing several different plasmids in each cell has the advantage that none of the expressed proteins is present at an extremely high level, which in some cases can make patterns difficult to recognize. On the other hand, the pattern generated by one plasmid may be obscured by those produced by many others. Therefore, the pattern of interest must be distinctive enough to be visible through this background. Probably because of this, our screen was most successful at identifying clones localized to single clear compartments in the cell. To find clones targeted to less easily distinguishable subcellular regions, variations on this visual screen may be more successful. For example, one could identify the structure of interest, perhaps with a targeted fusion to a color variant of GFP, and look for colocalization of transfected library fusions with the marked structure. Alternatively, one could take an approach in which only one clone was present per cell, either by starting with a completely subdivided library or by using a different method, like retroviral infection (21) at low multiplicity of infection, to introduce the library into cells. A combination of these modifications, in conjunction with automation, would make large-scale screening of the entire set of proteins of a cell possible.

It is likely that entire classes of proteins were missed in the screen because the GFP coding sequence had to be placed upstream of inserts derived from poly(A)-primed cDNAs. The GFP at the NH₂ terminus of the fusion protein is expected to block the function of many NH₂-terminal signal sequences, such as those used to target soluble and membrane proteins to the ER and mitochondria. Indeed, the mitochondrial and ER proteins we found did not contain this kind of signal sequence. Although proteins with NH₂-terminal targeting sequences might be found with a randomly primed cDNA library, poly(A) priming is more likely to give full-length clones and our results indicate that the most meaningful results were obtained with the entire coding sequence present.

Although some classes of proteins were not possible to find, we did identify proteins with a variety of topologies, from soluble to multispinning membrane proteins, targeted to many organelles. However, most clones contained cDNAs of previously identified proteins. The predominance of known proteins derives from their representation in the cDNA library. The library was not normalized, therefore, the frequency of a cDNA in the library reflects the abundance of its mRNA within the cell. A bias towards more abundant transcripts or proteins is not a

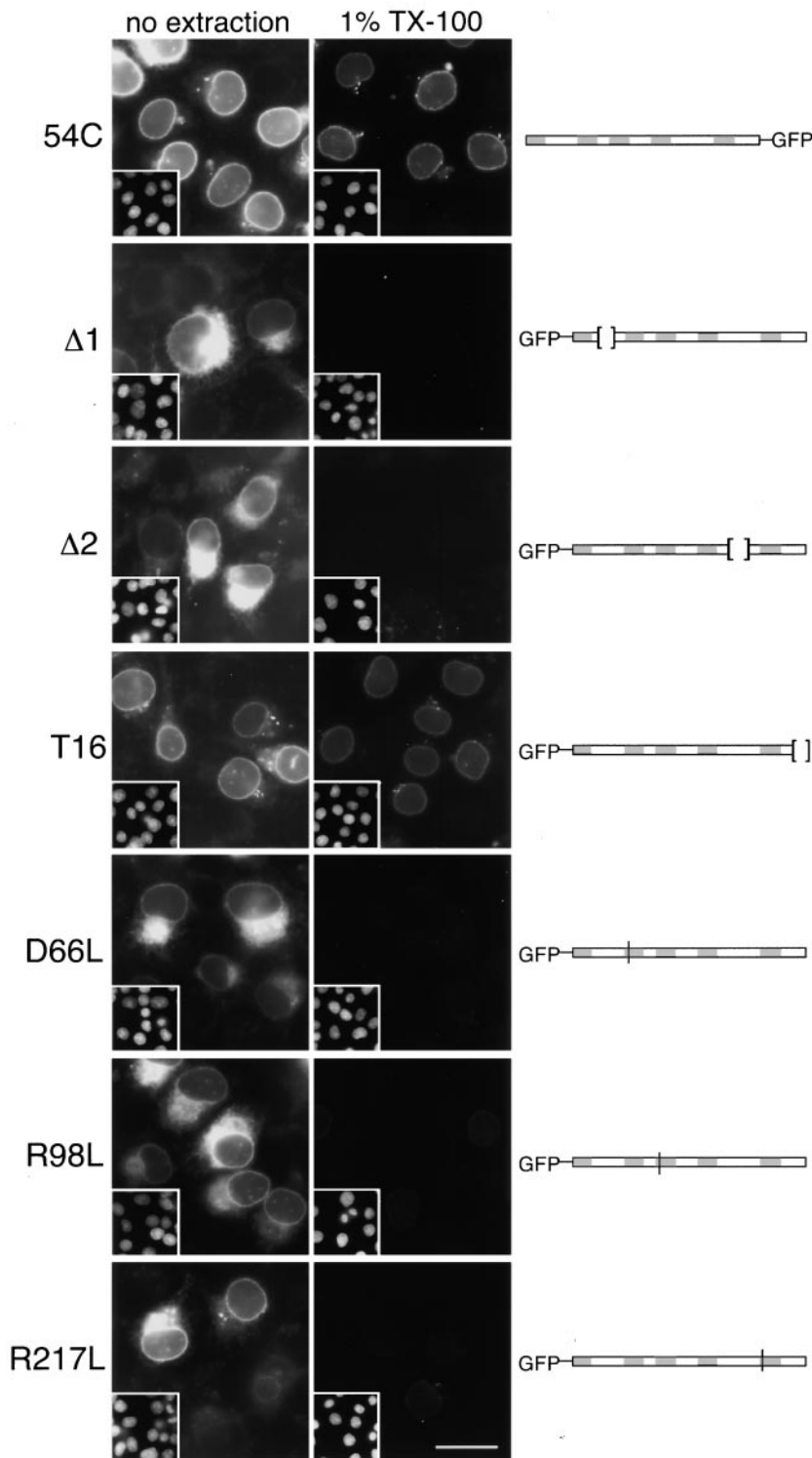


Figure 9. Localization and detergent sensitivity of GFP-nurim deletions and point mutants. BHK cells were transiently transfected with nurim-tagged with GFP at the COOH terminus rather than the NH₂ terminus (54C) or loop deletions ($\Delta 1$ and $\Delta 2$), truncation (T16), or point mutations (D66L, R98L, and R217L); diagrams of the mutants are included to the right with shading representing predicted transmembrane domains. Cells were either fixed immediately (no extraction) or extracted with 1% TX-100 before fixation (1% TX-100). GFP fluorescence is shown in large images and Hoechst staining to show nuclei in insets. All GFP images were taken at the same exposure and scaled identically. Bar, 20 μ m.

unique feature of this screen, so abundant molecules tend to be known. The visual screens in yeast avoided this problem by using libraries derived from genomic DNA. This approach is not practical in mammalian cells because of the frequency of introns. cDNA libraries can be normalized, although this is challenging, especially if full-length clones are desired. A normalized library containing long inserts would be very beneficial for future visual screens.

Using a visual screen to search for NE membrane proteins allowed us to identify a new kind of NE protein. Nurim, a 29-kD protein with five predicted transmembrane domains, does not belong to either of the two known classes of NE proteins. It is not targeted to nuclear pores and diverges in several respects from the lamin-associated NE membrane proteins. At the primary structure level, all members of the lamin-associated class have an

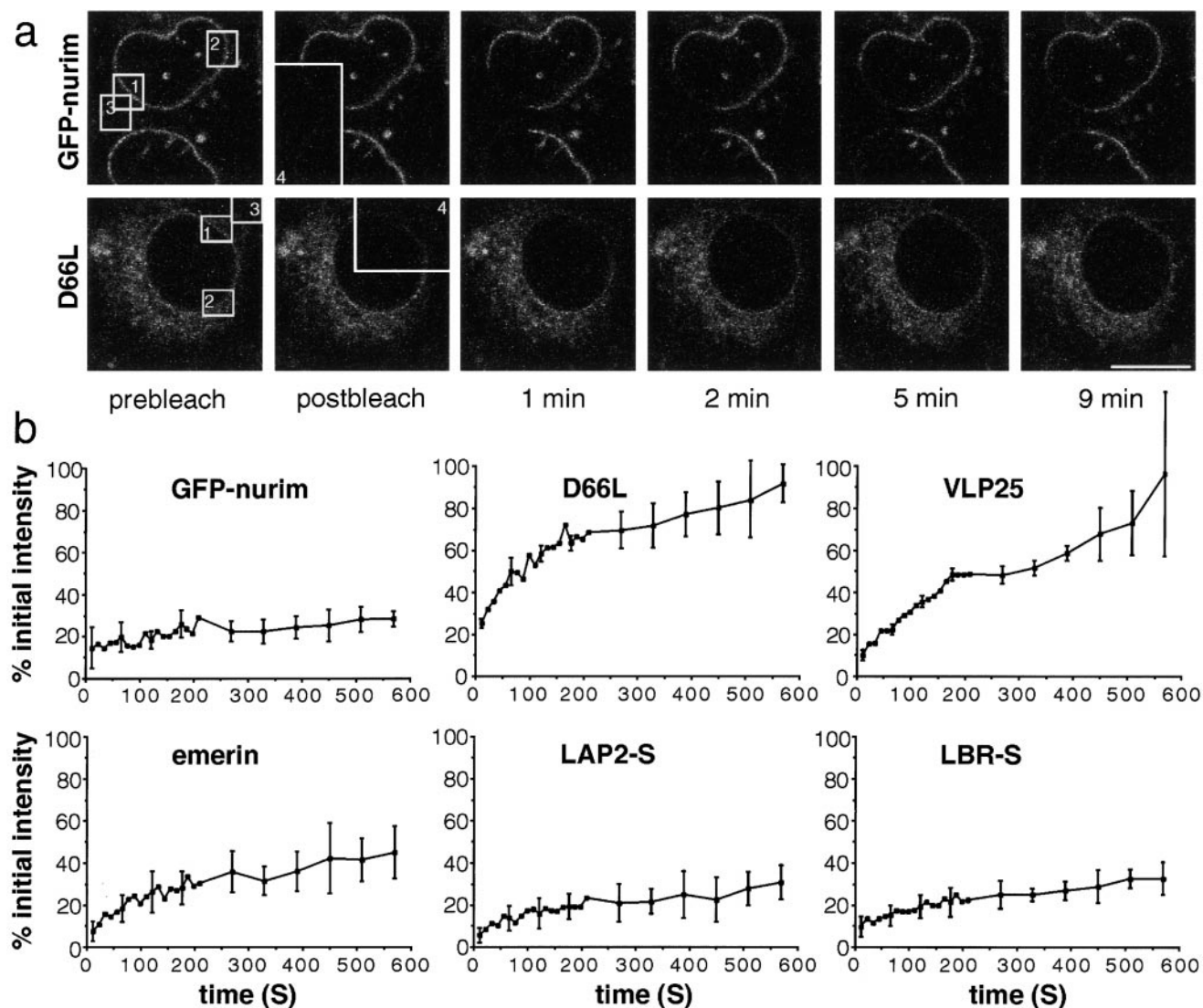


Figure 10. FRAP of GFP-fusions to NE and ER proteins. (a) BHK cells transiently transfected with GFP-nurim or point mutant D66L were imaged with a confocal microscope. A portion of the cell (box 4) was subjected to photobleaching and fluorescence recovery monitored by imaging every 11s for 220 s, and then every minute for 5 min. Examples of images of cells at various times after recovery are shown. (b) FRAP experiments were performed as in a for GFP-nurim, D66L, VLP25 (a GFP fusion to Sec61 β), YFP-emerin, LAP2-S, and LBR-S. The results from three bleached cells were quantitated and combined and the SD indicated with a bar (some of the SDs for early time points are not shown in the plots, but were similar to those shown). For quantitation, the total pixel intensity in a region of the cell that included the NE (a, box 1) was calculated. The intensity of an equivalent sized background area (box 3) was subtracted and, finally, the images were normalized for brightness using an unbleached region of the cell (box 2). Bar, 10 μ m.

NH₂-terminal nucleoplasmic domain of at least 200 amino acids, whereas nurim begins almost immediately with a transmembrane domain. The NH₂-terminal nucleoplasmic domain of the lamin-associated class can independently target membrane proteins to the NE, whereas we could not find an independent NE targeting signal in nurim. The results of mutagenesis suggested that nurim either forms a highly integrated structure or that multiple regions are required for targeting. In either case, charged residues in the transmembrane domains play an important role. Other experiments also distinguish nurim from the known proteins of the lamin-associated class. In nonmammalian cells (chicken fibroblasts) GFP fusions of LBR and LAP2 were

targeted to the NE, but GFP-nurim was not. Expression of human lamin A or C in chicken cells did not restore NE targeting of nurim. The mechanism of nurim targeting, thus, seems less conserved than that of the lamin-associated proteins.

Nurim may be among the most tightly bound membrane proteins of the nuclear envelope. FRAP experiments indicated that like other NE membrane proteins its diffusion is restricted in the NE. Similarly it shares with all known NE membrane proteins the resistance to extraction with TX-100 at physiological salt concentrations, but it cannot be extracted even at high salt concentrations when most known NE membrane proteins are solubilized. It is also in-

extractable with other detergents, including octyl-glucoside and C10-sucrose (not shown). Even after a series of treatments that leaves behind only the nuclear matrix, nurim remained visible at the nuclear periphery. This tight binding is clearly caused by its association with the nucleus since nurim was easily extractable when localized in the ER.

How nurim is targeted to the NE remains unclear. Although we have not directly ruled out binding to lamin B, we think it is unlikely to be targeted by direct binding to lamins because it does not contain a large nucleoplasmic domain and behaves differently from the lamin-associated NE proteins. We also consider it unlikely that nurim is bound to a specific complement of lipids in the NE that is different from that in the ER. A lipid-based mechanism should not be easily saturable while GFP-nurim is present in the ER even when moderately overexpressed. The resistance of nurim to a variety of nonionic detergents also argues against an association with only lipids. Instead, we favor the possibility that nurim is targeted to the NE by binding to another membrane protein.

While nurim is clearly localized to the NE, we do not know what role it might play there. For some of the known NE membrane proteins general functions have been identified, but their exact roles still remain to be defined. The membrane proteins within the nuclear pore belong to a large complex involved in regulating transport between nucleoplasm and cytoplasm, but their specific functions within this complex are not clear. The lamin-associated class of proteins has been suggested to play a structural role in maintaining the nuclear envelope (17). Microinjection of the lamin-binding regions of LAP2 inhibits nuclear growth in vivo (45). In vitro, addition of truncated LAP2 proteins to a nuclear assembly assay also inhibited nuclear growth (18). In addition, mutations in emerin can cause Emery-Dreifuss muscular dystrophy (5) and the same disease can be caused by mutations in lamin A/C (6), arguing that the defect of the nucleus in mutant cells is structural.

Other processes are likely to take place in the NE. In fact, the lamin-binding protein LBR also contains a domain with similarity to sterol reductases (33) and was recently shown to complement the ergosterol synthesis defect of a yeast lacking sterol C14 reductase (36). The NE is also implicated in several kinds of signaling. The unfolded protein response pathway from the ER to the nucleus involves a protein kinase, Ire1p, which, although not demonstrated to be targeted to the NE, must exert its function there (34). Nuclear calcium concentrations seem to be regulated independently from cytoplasmic calcium (25) and this is likely to involve NE proteins. The identification of a new kind of NE membrane protein that is probably neither involved in transport through nuclear pores nor in the maintenance of NE structure should provide a clue to additional functions of the NE.

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