Intracellular trafficking of MAN1, an integral protein of the nuclear envelope inner membrane

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Summary

MAN1 is an integral protein of the inner nuclear membrane that shares the LEM domain, a conserved globular domain of approximately 40 amino acids, with lamina-associated polypeptide (LAP) 2 and emerin. Confocal immuofluorescence microscopy studies of the intracellular targeting of truncated forms of MAN1 showed that the nucleoplasmic, N-terminal domain is necessary for inner nuclear membrane retention. A protein containing the N-terminal domain with the first transmembrane segment of MAN1 is retained in the inner nuclear membrane, whereas the transmembrane segments with the C-terminal domain of MAN1 is not targeted to the inner nuclear membrane. The N-terminal domain of MAN1 is also sufficient for inner nuclear membrane targeting as it can target a chimeric type II integral protein to this subcellular location. Deletion mutants of the N-terminal of MAN1 are not efficiently retained in the inner nuclear membrane. When the N-terminal domain of MAN1 is increased in size from ~50 kDa to ~100 kDa, the protein cannot reach the inner nuclear membrane. Fluorescence recovery after photobleaching experiments of MAN1 fused to green fluorescent protein show that the fusion protein is relatively immobile in the nuclear envelope compared with the endoplasmic reticulum of interphase cells, suggesting binding to a nuclear component. These results are in agreement with the 'diffusion-retention' model for targeting integral proteins to the inner nuclear membrane.

Keywords: Nuclear envelope, Inner nuclear membrane, LEM domain, Fluorescence recovery after photobleaching, Membrane proteins, Muscular dystrophy

Introduction

According to the 'diffusion-retention' model for the targeting of integral proteins to the inner nuclear membrane (Soullam and Worman, 1995), proteins synthesized on the rough endoplasmic reticulum (ER) membrane with cytoplasmic domains less than ~60 kDa can freely diffuse laterally between the ER to the inner nuclear membranes by moving along the nuclear pore membrane domain. The diffusional motilities of inner nuclear membrane proteins are significantly reduced at their final destination and they cannot readily diffuse back to the ER membrane (Ellenberg et al., 1997; Östlund et al., 1999). Retention in the inner nuclear membrane probably occurs as a result of protein-protein or protein-DNA interactions, either between the nucleoplasmic domains of these proteins and other nuclear structures or between transmembrane segments in the plane of the membrane. For example, the nucleoplasmic domain of the lamin B receptor (LBR), which confers inner nuclear membrane retention (Soullam and Worman, 1993), binds to B-type lamins (Ye and Worman, 1994) and human orthologues of *Drosophila* heterochromatin protein 1 (Ye and Worman, 1996; Ye et al., 1997). Similarly, the major nuclear envelope targeting domain of lamin-associated polypeptide (LAP) 2β coincides with its lamin binding region (Furukawa et al., 1998), and the nucleoplasmic domain of emerin, which mediates its inner nuclear targeting (Östlund et al., 1999; Tsuchiya et al., 1999), also binds to lamins (Clements et al., 2000). The transmembrane domains of LBR (Smith and Blobel, 1993; Soullam and Worman, 1995) and nurim (Rolls et al., 1999) also contribute to their immobilization in the inner nuclear membrane, probably via interactions with transmembrane segments of other resident proteins. This is also the case for gp210, an integral protein of the nuclear pore membrane domain (Wozniak and Blobel, 1992).

MAN1 is an integral protein of the inner nuclear membrane encoded by a gene on human chromosome 12q14 (Lin et al., 2000). It is predicted to have a nucleoplasmic, N-terminal domain followed by two hydrophobic segments and a nucleoplasmic, C-terminal tail. Indirect evidence suggests that MAN1 is associated with the nuclear lamina (Paulin-Levasseur et al., 1996). Protein sequence analysis reveals that MAN1 contains a conserved globular module of approximately 40 amino acids, which has been termed the LEM domain because it is found in LAP2, emerin and MAN1 (Lin et al., 2000). The LEM domain is composed mainly of two large parallel alpha helices in a fold similar to some bacterial dehydrogenase multienzyme complexes (Laguri et al., 2001; Wolff et al., 2001). Biochemical experiments have shown that the LEM domain binds to barrier-to-autointegration factor (Shumaker et al., 2001).

It is not known how MAN1 is targeted to or retained in the inner nuclear membrane. To investigate the intracellular trafficking of MAN1, we have transfected cells with plasmids that express different domains and chimeric constructs and examined their intracellular locations. We have also used

fluorescence recovery after photobleaching (FRAP) to measure the diffusion of a MAN1-green fluorescent protein (GFP) fusion protein in living cells. Our results are consistent with the 'diffusion and retention' model for MAN1 targeting to the inner nuclear membrane, with retention being mediated by its N-terminal domain.

Materials and Methods

Plasmid construction

Constructs that expressed FLAG-tagged polypeptides were made using pSVK3 (Amersham Pharmacia Biotech, Inc., Piscataway, NJ), which contains a multiple cloning site downstream from the SV-40 early promoter. Unless otherwise indicated, standard methods were used for cloning procedures (Sambrook et al., 1989). The cDNA sequence of MAN1 has been previously published (Lin et al., 2000) and is deposited in GenBank under accession number AF112299.

Plasmids FL, MAN538 and CT were constructed to express full-length MAN1, amino acids 1-538 (MAN1 nucleoplasmic, N-terminal domain followed the first hydrophobic segment) and the C-terminal tail with two hydrophobic segments of MAN1, respectively. The cDNAs for cloning were generated by polymerase chain reaction (PCR) (Saiki et al., 1987) using the Gene Amp PCR System 2400 (Applied Biosystems, Foster City, CA), with restriction endonuclease sites engineered at the 5′ ends of the oligonucleotide primers. The recombinant pSVK3 construct that encoded full-length MAN1 with a FLAG epitope tag at its N-terminus (plasmid FL) was used as a template to generate the other cDNAs. In most instances, an *Eco*RI site was engineered in the sense primer and a *Xho*I site in the antisense primer. PCR products were digested with the appropriate restriction endonucleases and ligated into pBFT4, which was digested with *Eco*RI and *Xho*I. pBFT4 is a pBluescript II KS- (Stratagene, La Jolla, CA) based plasmid containing a Kozak consensus sequence, ATG, and the FLAG coding sequence 5′ to the multiple cloning site. The resulting plasmids, which contained a *Spe*I site 5′ to the Kozak consensus sequence and a *Xho*I site 3′ to the cloned cDNA insert, were then digested with *Spe*I and *Xho*I and the isolated cassette ligated into pSVK3, which was digested with *Xba*I (an isoschizomer of *Spe*I) and *Xho*I.

Plasmids MAN476-CHL and MAN351-CHL were constructed to express FLAG-tagged proteins containing the first 476 and first 351 amino acids of MAN1, respectively, followed by the transmembrane domain and portion of the C-terminal domain of chicken hepatic lectin (CHL). The protein-coding cassette was isolated from the previously described plasmid LMBR-CHL (Soullam and Worman, 1993) by restriction endonuclease digestion with *Eco*RI and *Xho*I. This isolated cassette was ligated into pBFT4, which was also digested with *Eco*RI and *Xho*I. This plasmid was digested with *Eco*RI and *Bsp*EI, which cuts in the cDNA codon for amino acid 24 of CHL, and then the PCR products encoding amino acids 1-476 and amino acids 1-351 of MAN1, amplified using sense primers with an *Eco*RI site and antisense primers with a *Bsp*EI site, were ligated into it. These plasmids were then digested with *Spe*I and *Xho*I and the excised DNA ligated into pSVK3. To delete the coding region for amino acids 85-336 of MAN1 from plasmid MAN476-CHL, it was digested with *Not*I and the ends re-ligated to generate plasmid MANΔ85-336-CHL. To delete the nucleotides encoding amino acids 8-199 of MAN1 from plasmid MAN476-CHL, it was digested with *Nar*I and the ends re-ligated to generate plasmid MANΔ8-199-CHL.

Plasmid FG-CHL was constructed to express FLAG-tagged protein containing the transmembrane domain and a portion of the C-terminal domain of CHL. The pBFT4 construct containing the cDNA encoding the first 476 amino acids of MAN1 fused to CHL was digested with *Xma*I and *Bsp*EI and the compatible cohesive ends ligated. This construct was then digested with *Spe*I and *Xho*I and the excised DNA ligated into pSVK3.

To express a transmembrane protein that had a nucleoplasmic domain with a molecular mass of >60 kDa and the inner nuclear membrane targeting signal of MAN1, plasmid CMPK-MAN538 was constructed. This plasmid encoded a FLAG-tagged protein containing the first 538 amino acids of MAN1 fused to amino acids 17-476 of chicken muscle pyruvate kinase (CMPK) at the MAN1 N-terminus. This truncated form of CMPK lacks its mitochondrial signal sequence and is a soluble cytosolic protein (Frangioni and Neel, 1993). DNA encoding amino acids 17-476 of CMPK was amplified by PCR using plasmid p3PK (Frangioni and Neel, 1993) as template, using sense and antisense primers containing *Eco*RI sites. The PCR product, encoding amino acids 1-538 of MAN1, amplified using a sense primer with an *Eco*RI site and an antisense primer with an *Xho*I site, was ligated into pBFT4. The amplified product encoding amino acids 17-476 of CMPK was digested with *Eco*RI and ligated in-frame into the construct encoding amino acids 1-538 of MAN1. The resulting chimeric cDNA was excised by restriction endonuclease digestion with *Spe*I and *Xho*I and subcloned into pSVK3.

For studies of MAN1 fused to GFP, a FLAG-tagged construct containing amino acids 1-538 of MAN1 was fused, via its C-terminus, to the F64L, S65T, H231L variant of GFP. A cDNA fragment encoding a FLAG-tagged protein of the first 538 amino acids of MAN1 was isolated from plasmid MAN538 (see above) by restriction endonuclease digestion with *Sac*I. This fragment was ligated into pEGFP-N1 (CLONTECH Laboratories), which also was digested with *Sac*I. The resulting plasmid, MAN538-GFP, expressed the first 538 amino acids of MAN1 preceded by a FLAG epitope and followed by GFP. Plasmids MAN538Δ8-199-GFP and MAN538Δ85-336-GFP, which express GFP fusion proteins, are similar to MAN-538-GFP, with amino acids 8-199 and 85-336 of MAN1 deleted. To generate these plasmids, MAN538 was digested with *Not*I and *Nar*I, respectively, and the ends re-ligated to generate plasmids MAN538-Δ8-199 and MAN538-Δ85-336, respectively. The cDNA fragments were isolated from these two plasmids by restriction endonuclease digestion with *Sac*I and ligated into pEGFP-N1 that was digested with *Sac*I.

To confirm proper plasmid construction, cDNAs were sequenced using an ABI Prism 377 automated sequencer (Applied Biosystems).

Cell culture and transfection

COS-7 cells were grown in DME medium containing 10% fetal bovine serum and 2 mM L-glutamine. For transfection, cells were grown to 70-90% confluency on six-well plates, and transfected using LIPOFECT AMINE PLUS™ reagent (Life Technologies, Gaithersburg, MD), following the manufacturer's instructions. The cells were overlaid with the lipid-DNA complexes for 10-22 hours and allowed to grow in fresh medium for approximately 24-36 hours post-transfection. Cells were then washed with phosphate-buffered saline (PBS) and split into Chamber Slides or Chamber Coverglass (Nalge Nunc International Corp., Naperville, IL) and grown for an additional 12-24 hours before preparation for immunofluorescence microscopy or photobleaching.

Immunofluorescence microscopy

Transfected cells were washed three times with PBS and then fixed with methanol for 6 minutes at –20°C. The cells were permeabilized with 0.5% Triton X-100 in PBS for 2 minutes at room temperature, washed three times with 0.1% Tween-20 in PBS (Solution A) and incubated with the primary antibodies diluted in PBS containing 0.1% Tween-20 and 2% bovine serum albumin (Solution B) for 40 minutes at 37°C. Primary antibodies were anti-FLAG M5 monoclonal antibody (Sigma-Aldrich, St Louis, MO) used at a dilution of 1:200, anti-lamin B1 polyclonal antibody (Cance et al., 1992) used at a dilution of 1:1,000 and anti-signal-sequence-receptor α (anti-SSR-α) polyclonal antibodies (gift of Christopher Nicchitta, Duke University, Durham, NC) used at a dilution of 1:500. After washing four times

with Solution A, the cells were incubated with secondary antibodies diluted 1:200 in Solution B. Secondary antibodies used were rhodamine-conjugated goat anti-rabbit IgG (Biosource International, Camarillo, CA), fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Labs, Inc., West Grove, PA) and rhodamine-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Labs, Inc.). The cells were then washed four times with Solution A and three times with PBS. The slides were dipped in methanol, air-dried and the coverslips mounted using anti-fade mounting medium (Slowfade Light Antifade Kit, Molecular Probes, Eugene, OR).

Digitonin-permeabilization of cells was carried out as described previously (Adam et al., 1992). Transfected cells were washed three times with PBS and fixed with 2% paraformaldehyde in PBS for 30 minutes on ice. They were then washed three times with PBS and incubated with pre-cooled 40 mg/ml digitonin (Calbiochem, La Jolla, CA) in PBS for 10 minutes on ice. The cells were then washed and incubated with antibodies as described above, except that Tween-20 was excluded from the buffers and all steps were performed on ice.

Immunofluorescence microscopy was performed using a Zeiss LSM 410 confocal laser scanning system attached to a Zeiss Axiovert 100TV inverted microscope (Carl Zeiss, Inc., Thornwood, NY). Images were processed using PhotoShop software (Adobe Systems, Inc., San Jose, CA) on a Macintosh G3 computer (Apple Computer, Inc., Cupertino, CA).

Fluorescence photobleaching experiments

Fluorescence recovery after photobleaching was performed on the Zeiss LSM 410 confocal laser scanning system using a 488 nm line of a 15 mW Kr/Ar laser in conjunction with a 100× objective for optimum resolution or a 40× objective to achieve sufficient depth for bleaching in the optical axis (z). Command macros for programming the microscope for photobleaching experiments were downloaded from the Internet at http://dir.nichd.nih.gov/CBMB/pb2labob.htm. For qualitative experiments, the outlined box was photobleached at full laser power (100% power, 100% transmission) and recovery of fluorescence monitored by scanning the whole cell at low power (25% power and 1-3% transmission) in 10 second intervals. For quantitative experiments, the photobleached stripe was 2 μm wide and extended across the cell and through its entire depth. Fluorescence within the strip was measured at low laser power before the bleach and then photobleached with full laser power. Recovery was followed with low laser power at 2 second intervals until the intensity reached a steady plateau. Images were processed using PhotoShop software and average intensities were measured using NIH Image J software on a Macintosh G3 computer.

Other chemicals

Unless otherwise indicated, routine chemicals were obtained from either Fisher Scientific Co. (Pittsburgh, PA) or Sigma-Aldrich. Enzymes and enzyme buffers for DNA cloning were obtained from either Fisher Scientific Co. or New England Biolabs (Beverly, MA).

Results

The nucleoplasmic, N-terminal domain of MAN1 is necessary and sufficient for inner nuclear membrane targeting and retention

MAN1 is an integral protein of the inner nuclear membrane, with a nucleoplasmic N-terminal domain of 476 amino acids followed by a transmembrane segment, a luminal loop, a second putative transmembrane segment and a nucleoplasmic, C-terminal domain (Lin et al., 2000). The C-terminal domain is 252 amino acids, which is slightly longer than that reported previously by Lin et al. (Lin et al., 2000) (the corrected cDNA sequence of MAN1 can be found in GenBank under accession number AF112299). Our first objective was to determine the domain or domains of MAN1 responsible for its inner nuclear membrane targeting and retention. To accomplish this goal, we transfected cells with plasmid constructs to express MAN1, an endosome/ER protein CHL, various domains of MAN1 and MAN1-CHL chimeras. For consistent detection with monoclonal antibodies, the expressed proteins were engineered to contain a FLAG-epitope at their N-termini.

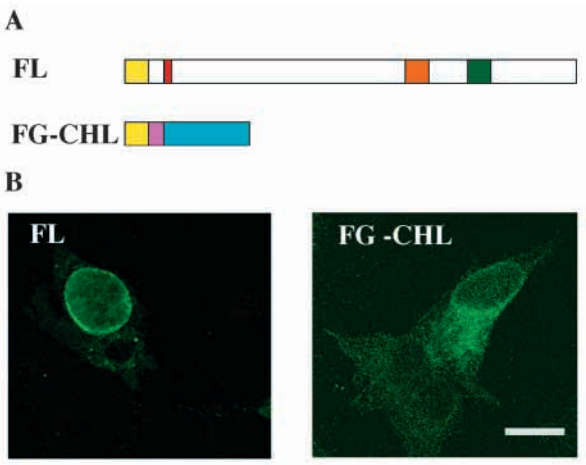

Fig. 1. Cellular localization of full-length MAN1 and CHL. (A) Proteins expressed by the plasmid constructs FL and FG-CHL, which were used to transfect COS-7 cells. Domains of proteins are represented as follows: yellow, FLAG-tag; white, nucleoplasmic, luminal and C-terminal domains of MAN1; red, LEM domain; orange, the first transmembrane segment of MAN1; dark green, the second transmembrane segment of MAN1; pink, transmembrane segment of CHL; light blue, luminal domain of CHL. The N-terminus of each protein is at the left. (B) Nuclear rim fluorescence characteristic of inner nuclear membrane labeling is seen for full-length MAN1 (FL) and a labeling pattern characteristic of outer nuclear membrane, ER and endosomes is seen for CHL (FG-CHL). Cells were fixed and immunostained with anti-FLAG monoclonal antibodies which were recognized by FITC-conjugated secondary antibodies. Bar, 10 μm.

To confirm the intracellular locations of full-length MAN1 and CHL, COS-7 cells were transfected with plasmids FL and FG-CHL (Fig. 1A). Full-length MAN1 was localized to the nuclear rim (Fig. 1B). CHL is a type II integral membrane protein localized to the ER, endosomes and plasma membrane. It has an N-terminal domain of 23 amino acids that faces the cytoplasm, a single transmembrane segment and a luminal, C-terminal domain of 160 amino acids (Chiacchia and Drickamer, 1984; Mellow et al., 1988). Strong labeling of the ER and other cytoplasmic membranes, along with weaker labeling of the plasma membrane, was seen in the transfected cells that expressed CHL (Fig. 1B). The fluorescence enhancement at the nuclear periphery in cells that expressed CHL was consistent with labeling of the outer nuclear membrane and ER, as the rough ER is concentrated around the nucleus and shares proteins with the contiguous outer nuclear membrane.

To determine the domain of MAN1 responsible for its inner nuclear membrane localization, we transiently transfected COS-7 cells with plasmids expressing truncated and chimeric

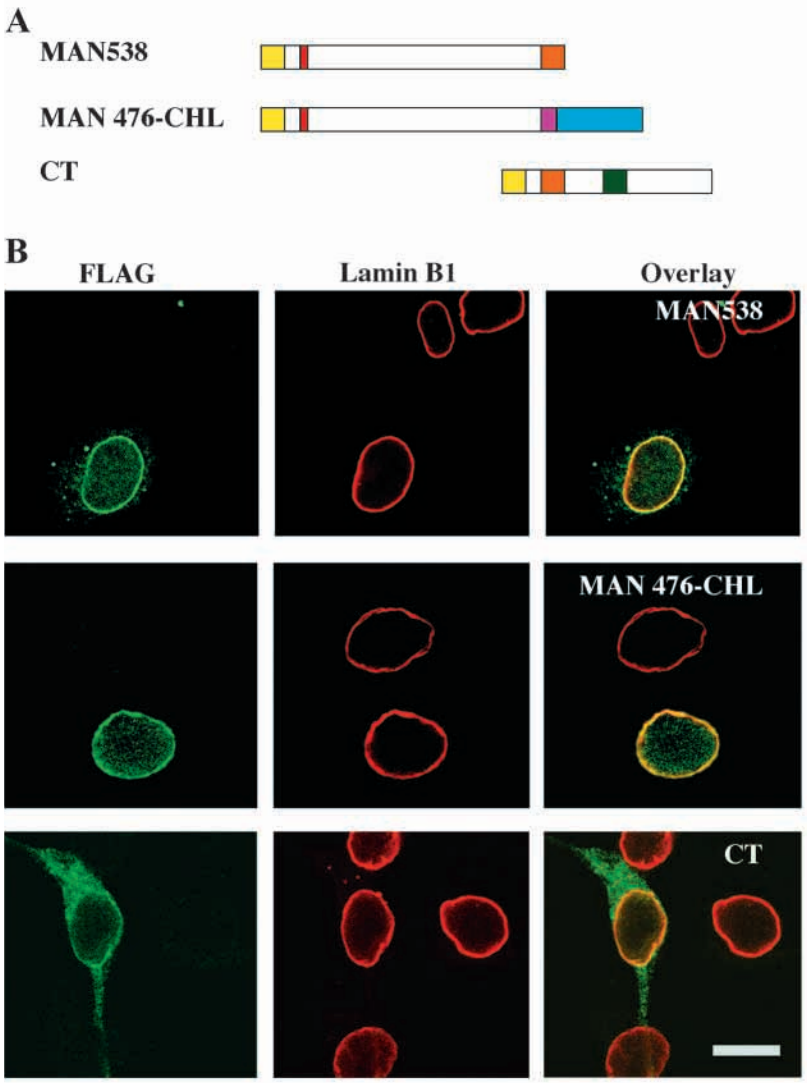

Fig. 2. The N-terminal domain of MAN1 contains a nuclear envelope targeting signal. (A) Proteins expressed by the plasmid constructs MAN538, MAN476-CHL and CT, which were used to transfect COS-7 cells. Domains of proteins are represented by: yellow, FLAG-tag; white, nucleoplasmic, luminal and C-terminal domains of MAN1; red, LEM domain; orange, the first transmembrane segment of MAN1; dark green, the second transmembrane segment of MAN1; pink, transmembrane segment of CHL; light blue, luminal domain of CHL. The N-terminus of each protein is on the left. (B) Cellular localizations of the N-terminal domain with the first transmembrane segment of MAN1 (MAN538), the N-terminal domain of MAN1 (amino acids 1-476) fused to truncated CHL (MAN476-CHL) and the two transmembrane segments and nucleoplasmic, C-terminal domain of MAN1 (CT) as determined by laser scanning confocal immunofluorescence microscopy. Antibodies used were anti-FLAG monoclonal antibodies recognized by FITC-conjugated secondary antibodies (green, left panels labeled FLAG) and polyclonal antibodies against lamin B1 recognized by rhodamine-conjugated secondary antibodies (red, middle panels labeled Lamin B1). The panels on the right show an overlay of the FITC and rhodamine channels, with areas of overlap appearing yellow. Bar, 10 μm.

forms of the protein (Fig. 2A). COS-7 cells were transfected with plasmid MAN538, which encodes the nucleoplasmic, N-terminal domain and the first transmembrane segment of MAN1. This protein was detected at the nuclear rim by immunofluorescence microscopy, showing colocalization with lamin B1, a marker for the lamina and inner nuclear membrane, consistent with an inner nuclear membrane localization (Fig. 2B). MAN1 devoid of its N-terminal nucleoplasmic domain was localized to the ER and possibly endosomes (Fig. 2B, CT), showing that the N-terminal domain is necessary for inner nuclear membrane targeting. To determine if the N-terminal domain of MAN1 can function as a nuclear envelope targeting signal for another integral membrane protein, it was attached to the transmembrane segment of CHL. In cells expressing a FLAG-tagged chimeric protein containing the N-terminal domain of MAN1 fused to the N-terminal side of the transmembrane segment of the CHL, nuclear rim fluorescence without ER labeling was observed, consistent with inner nuclear membrane localization (Fig. 2B). Hence, the N-terminal domain of MAN1 can function as a nuclear envelope targeting signal sufficient to direct an integral membrane protein synthesized on the ER to the inner nuclear membrane.

To confirm that the N-terminal domain of MAN1 attached to a transmembrane segment localized to the inner as opposed to the outer nuclear membrane, the plasma membranes of cells expressing the first 538 amino acids of MAN1 (N-terminal domain and first transmembrane segment) with a FLAG epitope were selectively permeabilized with digitonin (Adam et al., 1992). To confirm further that the FLAG-tagged protein was detected at the intact nuclear rim but inaccessible to anti-FLAG antibodies, cells expressing the FLAG-tagged protein containing the first 538 amino acids of MAN1 fused to GFP (Fig. 3A) were treated with Triton X-100 or digitonin. The endogenous fluorescence from GFP was then visualized simultaneously to antibody labeling with anti-FLAG antibodies recognized by rhodamine-conjugated secondary antibodies (Fig. 3B). Cells in which the nuclear envelopes were permeabilized with Triton X-100 showed an overlap of the signals from the rhodamine channel and the GFP channel. In cells permeabilized with digitonin, green fluorescence was detected at the nuclear rim but it was not labeled with anti-FLAG antibodies. This experiment clearly showed that the protein containing the N-terminal domain of MAN1 and its first transmembrane segment was located in the inner nuclear membrane.

We next attempted to determine if a smaller portion of the N-terminal domain of MAN1 could mediate inner nuclear membrane targeting and retention. COS-7 cells were transfected with plasmids MANΔ8-199-CHL, MANΔ85-336-CHL and MAN351-CHL, from which amino acids 8-199, 85-336 and 352-476, respectively, were deleted from the protein containing the MAN1 N-terminal domain followed by CHL (Fig. 4A). In cells transfected with these plasmids, the expressed proteins were detected in the ER and nuclear envelope of transfected cells (Fig. 4B). However, unlike full-length MAN1 (Figs 1, 2), fluorescence labeling with these

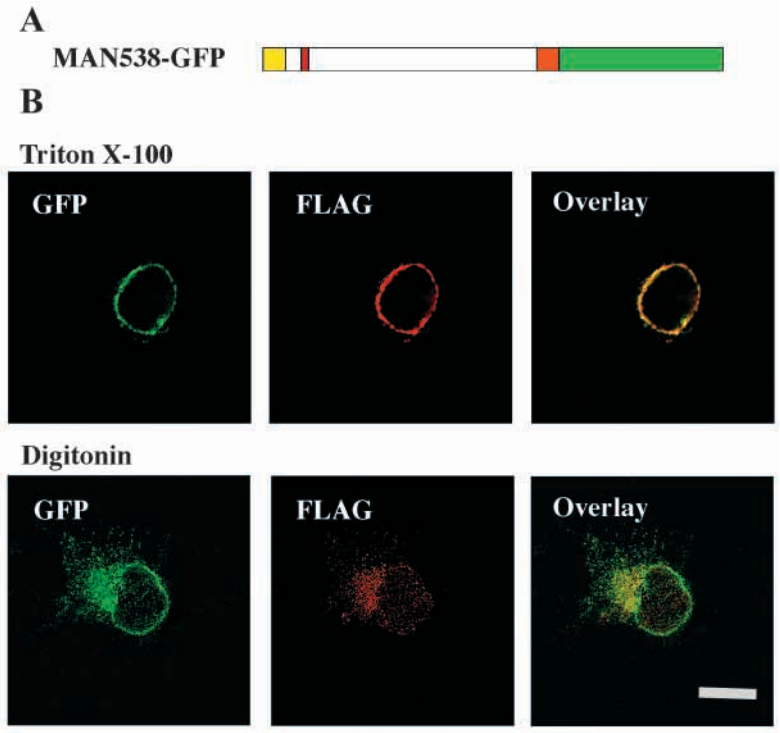

Fig. 3. The nucleoplasmic, N-terminal domain of MAN1 plus its first transmembrane domain is localized to the inner nuclear membrane using digitonin-permeabilized cells. (A) Protein expressed by the plasmid construct MAN538-GFP, which was used to transfect COS-7 cells. Domains of the protein are represented by: yellow, FLAG-tag; white, nucleoplasmic, luminal and C-terminal domains of MAN1; red, LEM domain; orange, the first transmembrane segment of MAN1; green, GFP. The N-terminus of the protein is on the left. (B) Fluorescence microscopic analysis of COS-7 cells that were transfected with plasmid expressing the FLAG-tagged N-terminal domain and first transmembrane segment of MAN1 fused to GFP. The cells were permeabilized with Triton X-100 (upper panels, labeled Triton X-100) or ice-cold digitonin (lower panels, labeled Digitonin) and were then labeled with anti-FLAG monoclonal antibody. The left panels (GFP) show fluorescence from GFP (green), the middle panels (FLAG) show signal from anti-FLAG monoclonal antibodies recognized by rhodamine conjugated secondary antibodies (red) and the right panels (Overlay) show an overlay of the GFP and FLAG panels, with areas of overlap appearing yellow. Bar, 10 μm.

internally truncated constructs was never exclusive to the nuclear envelope. Fluorescence labeling of the ER and nuclear envelope does not exclude the possibility that some of the expressed protein is in the inner nuclear membrane or nuclear pore membranes; however, a large portion of protein is in the ER. Therefore, even if some portion of these chimeric proteins reaches the inner nuclear membrane, their retention signals are significantly weaker than those present in proteins containing the whole N-terminal domain of MAN1. These results suggest that the entire nucleoplasmic, N-terminal domain of MAN1 is essential for efficient inner nuclear membrane retention.

Enlargement of the nucleoplasmic, N-terminal domain of MAN1 prevents its inner nuclear membrane targeting

The results presented so far suggest that N-terminal of MAN1 can target an integral membrane protein synthesized on the ER to the inner nuclear membrane. For this to occur, the protein would presumably have to diffuse through the nuclear pore complexes. The nuclear pore complex contains a central channel, through which soluble proteins are thought to be actively transported, as well as lateral channels with diameters of 10 nm, through which proteins with a molecular mass of <60 kDa can presumably diffuse (Hinshaw et al., 1992). The lateral channels are located adjacent to the pore membrane domain (Hinshaw et al., 1992), and the cytoplasmically exposed domains of transmembrane proteins would have to pass through them en route to the inner nuclear membrane. To test if a transmembrane protein targeted by the N-terminal domain of MAN1 gained access to the inside of the nucleus via the lateral channels of the nuclear pore complexes, we enlarged the cytoplasmically synthesized domain of a MAN1 polypeptide that was normally localized in the inner nuclear membrane.

We fused the N-terminal domain of MAN1 with its first transmembrane segment to truncated CMPK at MAN1's N-terminus (Fig. 5A). This truncated CMPK is a non-membrane protein localized to the cytosol (Frangioni and Neel, 1993; Soullam and Worman, 1995). The molecular mass of the cytoplasmically synthesized domain of this CMPK-MAN1 chimeric protein preceding the transmembrane segment was increased to approximately 100 kDa. This chimeric protein did not concentrate in the inner nuclear membrane but remained primarily in the ER (Fig. 5B), despite containing an inner nuclear membrane targeting domain. This implies that MAN1 diffuses from the ER to the inner membrane through the lateral channels of the nuclear pore complexes.

Diffusional mobility of MAN1 in the inner nuclear and ER membranes

To examine the diffusion of MAN1 in the inner nuclear and ER membranes, we performed FRAP experiments using a protein with the first 538 amino acids of MAN1 (nucleoplasmic, N-terminal domain plus first transmembrane segment) fused at its C-terminus to GFP (Fig. 3A). In cells overexpressing this GFP fusion protein at a relatively high level, it 'backed up' in the ER, probably when its binding or retention sites in the nuclear membrane were filled. This allowed us to measure the protein's diffusion in the inner nuclear and ER membranes, as we have done previously for LBR (Ellenberg et al., 1997) and emerin (Östlund et al., 1999). In cells expressing the MAN1-GFP fusion protein, the bleached nuclear envelope area did not regain full fluorescence intensity 290 seconds after photobleaching (Fig. 6A), whereas in the bleached ER area, fluorescence recovered quickly, regaining approximately 70% of its original fluorescence intensity after about 60 seconds (Fig. 6B). Approximately 80% of the nuclear envelope fluorescence recovered 10 minutes after photobleaching.

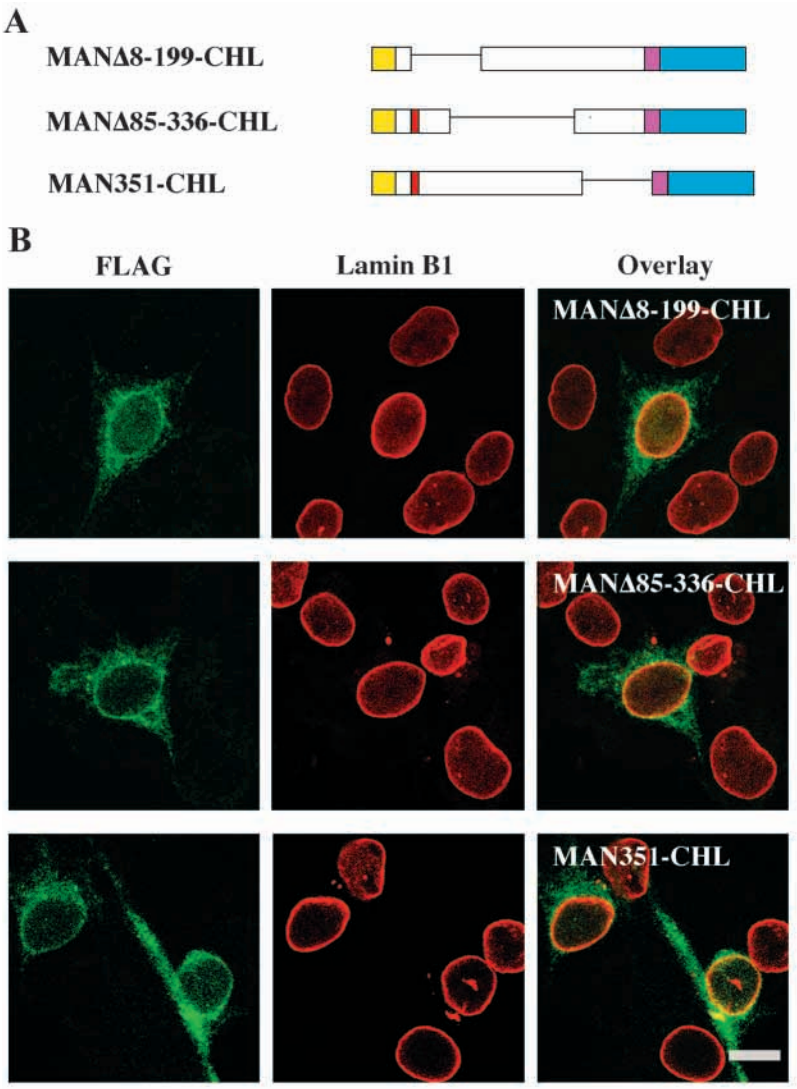

Fig. 4. The entire N-terminal domain of MAN1 is necessary for efficient inner nuclear membrane targeting and retention. (A) Proteins expressed by the plasmid constructs MANΔ8-199-CHL, MANΔ85-336-CHL and MAN351-CHL, which were used to transfect COS-7 cells. Domains of the proteins are represented by: yellow, FLAG-tag; white, nucleoplasmic domain of MAN1; red, LEM domain; pink, transmembrane segment of CHL; light blue, luminal domain of CHL. Thin lines indicate portions of MAN1 deleted from each protein. The N-terminus of each protein is on the left.
(B) Immunofluorescence images from laser scanning confocal immunofluorescence microscopy showing localization of MAN1-CHL chimeric proteins from which amino acids 8-199 (MANΔ8-199-CHL), 85-336 (MANΔ85-336-CHL) and 351-476 (MAN351-CHL) of MAN1 were deleted. Antibodies used were anti-FLAG monoclonal antibodies recognized by FITC-conjugated secondary antibodies (green, left panels labeled FLAG) and polyclonal antibodies against lamin B1 recognized by rhodamine-conjugated secondary antibodies (red, middle panels labeled Lamin B1). The panels to the right show an overlay of the FITC and rhodamine channels, which areas of overlap appearing yellow. Bar, 10 μm.

Quantitative FRAP experiments were performed to measure the diffusional mobility of the MAN1-GFP fusion protein in the nuclear envelope and ER membranes. Normalized mean fluorescence intensities in a 2 μm bleached strip before and after the photobleach were plotted versus time to determine the diffusion constants (D), using previously described methods (Ellenberg et al., 1997), in the two different membrane pools (Fig. 7). D was 0.28±0.04 μm^2/second for the ER pool and 0.12±0.02 μm^2/second for the nuclear envelope pool (mean±s.d.). These results showed that MAN1 was significantly less mobile in the inner nuclear membrane compared with the ER (Table 1).

We also examined the diffusional mobilities of two GFP fusion proteins of the first 538 amino acid of MAN1 from which amino acids 8-199 (MAN538Δ8-199-GFP) and 85-336 (MAN538Δ85-336-GFP) were deleted. Deletion of either of these two stretches of amino acids prevents the N-terminal domain of MAN1 from functioning as an efficient nuclear targeting signal (Fig. 4). The measured diffusion constant in the ER was 0.26±0.02 μm^2/second for MAN538Δ8-199-GFP and 0.29±0.02 μm^2/second for MAN538Δ85-336-GFP, not significantly different than for the GFP fusion protein containing the entire N-terminal domain of MAN1. An immobile fraction in the nuclear envelope could not be measured because of the predominant ER/outer nuclear membrane localization that was always present. These findings further indicate that MAN1 can diffuse rather freely in the ER and that the entire N-terminal domain is necessary for efficient inner nuclear membrane retention.

Discussion

Targeting of integral proteins to the inner nuclear membrane

Several other studies have examined the targeting of integral proteins to the inner nuclear membrane (Smith and Blobel, 1993; Soullam and Worman, 1993; Soullam and Worman, 1995; Furukawa et al., 1995; Furukawa et al., 1998; Ellenberg et al., 1997; Cartegni et al., 1997; Ellis et al., 1998; Östlund et al., 1999; Rolls et al., 1999; Tsuchiya, 1999). Inner nuclear membrane targeting signals have been shown to reside in the nucleoplasmic domains of LBR (Soullam and Worman, 1993), LAP2β (Furukawa et al., 1995) and emerin (Östlund et al., 1999; Tsuchiya et al., 1999). The diffusional mobilities of LBR (Ellenberg et al., 1997), emerin (Östlund et al., 1999) and nurim (Rolls et al., 1999) have also been measured using FRAP

Table 1. Diffusion constants (D) for the first 538 amino acids of MAN1 fused to GFP in the ER and nuclear envelope

Membrane	D (μm^2/second)	*n*
ER	0.28±0.04	6
Nuclear envelope	0.12±0.02	6

t-test shows that the D value for MAN1-GFP in the ER is significantly larger than that in the nuclear envelope (t=8.89, P<0.01). Results are mean±s.d. for n=6 separate determinations.

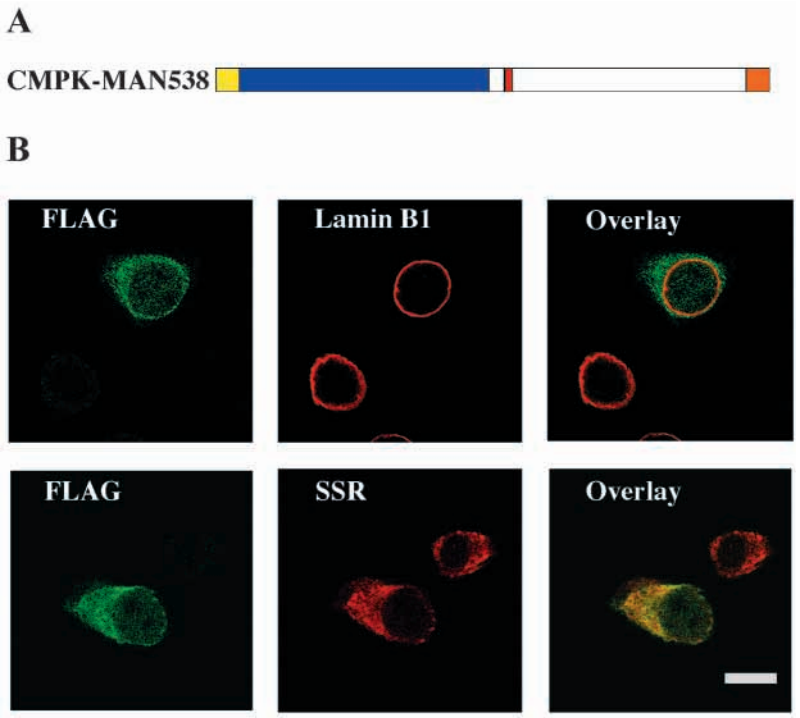

Fig. 5. Size of the nucleocytoplasmic domain of MAN1 affects its inner nuclear membrane targeting. (A) Protein expressed by the plasmid CMPK-MAN538, which was used to transfect COS-7 cells. Domains of the protein are represented by: yellow, FLAG-tag; dark blue, truncated CMPK; white, nucleoplasmic domain of MAN1; red, LEM domain; orange, the first hydrophobic transmembrane segment of MAN1. The N-terminus of the protein is on the left. (B) Immunofluorescence images from laser scanning confocal immunofluorescence microscopy showing localization of truncated CMPK fused to the N-terminal domain and first transmembrane segment of MAN1. Antibodies were anti-FLAG monoclonal antibodies recognized by FITC-conjugated secondary antibodies (green, upper and lower left panels labeled FLAG), polyclonal antibodies against lamin B1 (labeled Lamin B1) or against SSR-alpha (labeled SSR), which is a marker for ER protein, recognized by rhodamine-conjugated secondary antibodies (red, middle panels). The right panels show an overlay of the FITC and rhodamine channels, with areas of overlap appearing yellow. Bar, 10 μm.

and they are significantly reduced in the inner nuclear membrane compared with the ER membranes. These findings are consistent with the 'diffusion-retention' model for inner nuclear membrane targeting, as originally proposed by Soullam and Worman (Soullam and Worman, 1995). In this model, integral proteins synthesized on the ER membrane can diffuse freely in the ER and continuous (and probably identical) outer nuclear membrane. These proteins can reach the inner nuclear membrane via the nuclear pore membranes, diffusing through the lateral channels of the nuclear pore complexes. Once immobilized in the inner nuclear membrane, resident integral membrane protein cannot freely diffuse out to the outer nuclear membrane, as supported by measurements made using fluorescence loss in photobleaching of GFP fusions (Ellenberg et al., 1997; Östlund et al., 1999).

The results of our present studies on MAN1 are also consistent with its targeting to the inner nuclear membrane by a 'diffusion-retention' mechanism. Its nucleoplasmic, N-terminal domain mediates accumulation in the inner nuclear membrane after synthesis on the ER membrane. The N-terminal domain of MAN1 with its first transmembrane segment diffuses rather freely in the ER membrane, but its lateral diffusion constant is decreased by greater than half, from D=0.28 μm^2/second in the ER to D=0.12 μm^2/second, in the inner nuclear membrane. These values are similar to those reported for emerin of D=0.32 μm^2/second in the ER and D=0.10 μm^2/second in the nuclear envelope (Östlund et al., 1999). By contrast, LBR appears to be more immobile in the nuclear envelope compared to MAN1 and emerin (Ellenberg et al., 1997).

The N-terminal domain of LBR binds to B-type lamins (Ye and Worman, 1994) and human orthologues of *Drosophila* heterochromatin protein 1 (Ye and Worman, 1996; Ye et al., 1997). The N-terminal domain of LAP2β similarly binds to lamins (Furukawa et al., 1998) and chromatin (Foisner and Gerace, 1993) and the inner nuclear membrane targeting domain localizes with its lamin binding domain (Furukawa et al., 1998). The N-terminal domain of emerin binds to nuclear lamins (Clements et al., 2000) and probably one or more chromatin components based on the colocalization of different portions of emerin with different chromosomal regions during mitosis (Haraguchi et al., 2000). Specific nuclear proteins that bind to MAN1 have not yet been identified; however, MAN1 cofractionates with nuclear lamins, suggesting an interaction with the lamina (Paulin-Levasseur et al., 1996). Like emerin, MAN1 also contains a LEM domain (Lin et al., 2000; Wolff et al., 2001), which binds to the predominantly nuclear protein barrier-to-autointegration factor (Shumaker et al., 2001). Most or all of the 476 amino acid N-terminal domain of MAN1 is necessary for efficient inner nuclear membrane retention, suggesting that binding to more than one nuclear component is required for concentration there. Alternatively, a complex quaternary structure of the entire N-terminal domain may be required for binding to one nuclear structure.

Transmembrane domains of some integral membrane proteins also contribute to their localization in the inner and pore membrane domains of the nuclear envelope. This may occur as a result of oligomerization in the plane of the membrane or differential retention in membranes of varying thickness (Bretscher and Munro, 1993; Nilsson and Warren, 1994). Transmembrane segments contribute to the inner nuclear membrane localization of LBR (Smith and Blobel, 1993; Soullam and Worman, 1995) and nurim (Rolls et al., 1999) and the pore membrane targeting of glycoprotein gp210, whose cytoplasmic tail and transmembrane segment can mediate targeting to this location (Wozniak and Blobel, 1992). However, proteins very similar in structure to LBR in their transmembrane segments but lacking hydrophilic N-terminal domains are localized predominantly to the ER (Holmer et al., 1998), suggesting that the N-terminal domain of LBR is its most important targeting signal. By contrast, the

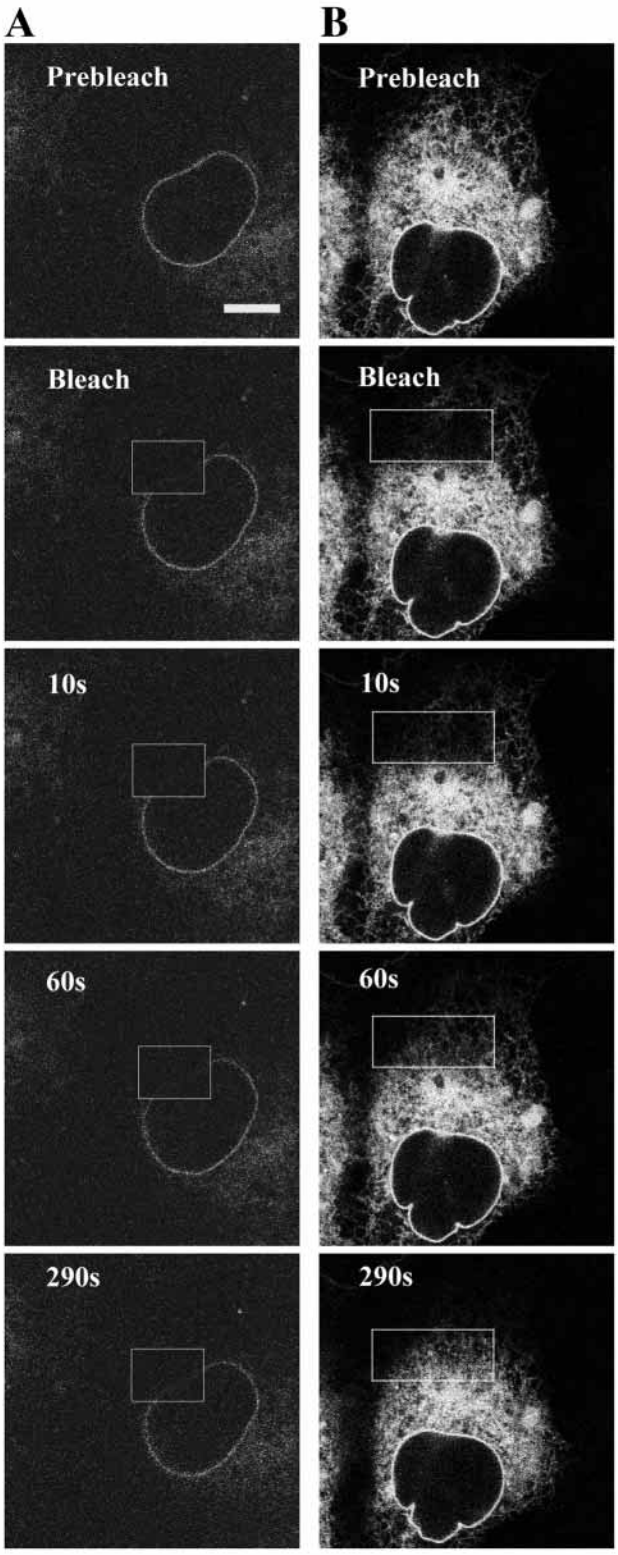

Fig. 6. The lateral diffusional mobility of MAN1 is decreased in the nuclear envelope compared with the ER. Confocal fluorescence studies of localization and mobilities of the MAN1-GFP fusion protein encoded by plasmid MAN538-GFP (see legend to Fig. 3) measured by qualitative FRAP, showing photobleach recovery in (A) nuclear envelope membranes and in (B) ER membranes. The fluorescence in the boxed regions was bleached and the fluorescence recovery is shown at 10, 60 and 290 seconds after the bleaching. Bar, 5 μm.

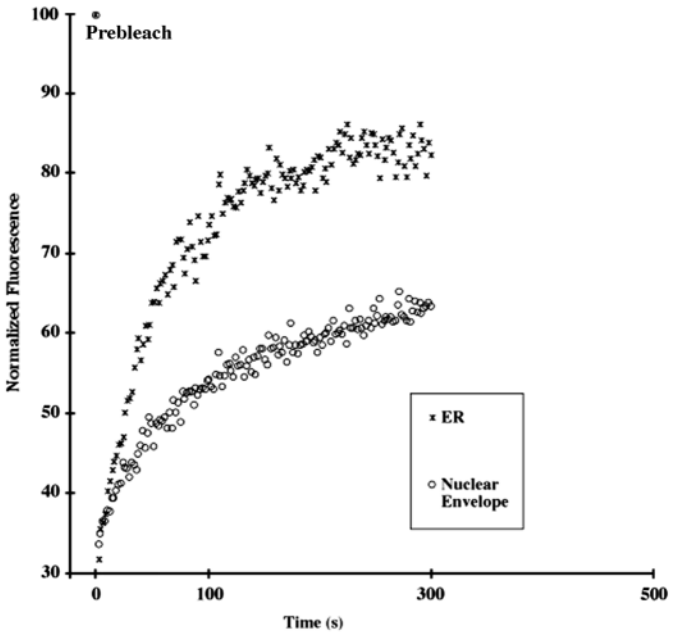

Fig. 7. Comparison of FRAP experiments for the MAN1-GFP fusion protein (see Fig. 6) in the ER and nuclear envelope membranes. Fluorescence intensities in recovery after photobleaching are plotted versus time for the fusion protein in the ER (x) and nuclear envelope (o). Data points were obtained at 2 second intervals. The curves displayed kinetics allowing for the determination of diffusion constants for MAN1-GFP in the ER and nuclear envelope. Fluorescence intensity was normalized to prebleach intensity and corrected for total loss of fluorescence because of the high-energy laser bleach to I_0=100 (normalized prebleach intensity).

transmembrane segments of nurim (Rolls et al., 1999) and gp210 (Wozniak and Blobel, 1992) may be their major targeting determinants. For the inner nuclear membrane proteins emerin (Östlund et al., 1999; Tsuchiya et al., 1999) and LAP2β (Furukawa et al., 1995) and the nuclear pore membrane protein POM121 (Söderqvist et al., 1997), the transmembrane segments do not mediate targeting to a significant degree. This is also the case for MAN1.

Is the inner nuclear membrane a specialized domain of the ER?

The ‘diffusion-retention’ model for inner nuclear membrane protein targeting and the observed morphology of the nuclear envelope imply that the inner nuclear membrane is actually a specialized domain of the ER. The outer nuclear membrane, which contains ribosomes on its outer surface, is directly continuous with the rough ER and similar or identical to it in composition (Amar-Costesec et al., 1974; Pathak et al., 1986). Viral integral membrane proteins synthesized on the ER are also found in the outer, pore and inner membrane domains of the nuclear envelope (Bergmann and Singer, 1983; Torrisi and Bonnatti, 1985). Studies using FRAP (Ellenberg et al., 1997; Östlund et al., 1999; Rolls et al., 1999), including the present study, have shown that the lateral diffusion of integral proteins is significantly different in the ER/outer nuclear membrane and inner nuclear membrane, with the diffusion of resident proteins being significantly decreased in the inner nuclear membrane.

The inner nuclear membrane is separated from the remainder of the ER by the nuclear pore complexes, which form an immobile network and have a very low turnover in live mammalian cells (Daigle et al., 2001). The nuclear lamina, a meshwork of intermediate filaments, is associated with the inner nuclear membrane, and FRAP experiments have shown that the peripheral lamina is also relatively immobile in interphase cells (Broers et al., 1999; Moir et al., 2000). The pore complexes and the lamina are also associated in cells (Aaronson and Blobel, 1975), further contributing to the establishment of a 'rigid' macromolecular structure supporting the inner nuclear membrane. The adjacent chromatin, another immobile structure in the interphase nucleus (Marshall et al., 1997), may also contribute to the different properties of the inner nuclear membrane compared with the rest of the ER. Most integral proteins are probably localized to the inner nuclear membrane primarily because they are immobilized there by binding to the adjacent rigid lamina and chromatin. In mitosis, integral proteins such as LBR and emerin, which are relatively immobile in the inner nuclear membrane in interphase, diffuse more freely in the ER (Ellenberg et al., 1997; Haraguchi et al., 2000). This suggests that the inner nuclear membrane loses its differentiation from the remainder of the ER in mitosis when the nuclear lamina depolymerizes, the pore complexes disassemble and the chromatin condenses into chromosomes.

Although the inner and outer nuclear membranes are directly continuous via the pore membrane, the pore complexes present a topological barrier to the movement of MAN1 and other integral proteins from their site of synthesis on the ER membrane to the inner nuclear membrane. Structural studies have indicated that aqueous channels of approximately 10 nm are present at the periphery of the pore complex immediately adjacent to the pore membrane (Hinshaw et al., 1992). A globular protein of approximately 60 kDa can freely diffuse through an aqueous channel of this size. When truncated CMPK was fused to the MAN1 N-terminal domain, resulting in a domain of approximately 100 kDa preceding the transmembrane segment, it was retained in the ER. Similar results have been observed when the nucleoplasmic, N-terminal domain of LBR (Soullam and Worman, 1995) was enlarged. These results show that the lateral channels of the nuclear pore complexes provide barriers that prevent integral proteins with cytoplasmically synthesized domains greater than ~60 kDa from reaching the inner nuclear membrane. Along these lines, all isoforms of the six integral membranes proteins definitely localized to the inner nuclear membrane in interphase – LBR, LAP1, LAP2, emerin, nurim and MAN1 – have nucleocytoplasmic domains with molecular masses of <60 kDa. Hence, the nuclear pore complexes play an important role in differentiating the inner nuclear membrane from the rest of the ER.

Inner nuclear membrane protein trafficking in inherited diseases

Inherited mutations in inner nuclear membrane proteins cause human diseases. Mutations in emerin were first shown to cause X-linked Emery-Dreifuss muscular dystrophy (Bione et al., 1994). Most of these mutations lead to a loss of emerin from all cells, including skeletal and cardiac muscle (Manilal et al., 1996; Nagano et al., 1996). A wide range of mostly missense and deletion mutations in nuclear lamins A and C, which interact with emerin (Clements et al., 2000), causes an autosomal dominantly inherited form of Emery-Dreifuss muscular dystrophy (Bonne et al., 1999; Bonne et al., 2000). In addition, missense and deletion mutations cause two phenotypically overlapping disorders: dilated cardiomyopathy with conduction deficit (Fatkin et al., 1999) and limb girdle muscular dystrophy type 1b (Muchir et al., 2000). Localized mutations in a specific region of the C-terminal tail domain of lamins A and C cause autosomal dominant Dunnigan-type familial partial lipodystrophy (Cao and Hegele, 2000; Shackleton et al., 2000; Speckman et al., 2000).

How do mutations in emerin and lamins A and C cause tissue-specific human diseases? This question has been the subject of considerable recent experimental attention and theoretical speculation but remains unanswered (Emery, 2000; Flier, 2000; Morris, 2000; Nagano and Arahata, 2000; Hutchison et al., 2001; Wilson et al., 2001). Our current results show that the LEM domain-containing protein MAN1, like emerin (Östlund et al., 1999), is immobilized in the inner nuclear membrane in interphase. In cells from *Lmna* knockout mice that do not express lamins A and C, some emerin is lost from the nuclear envelope and mislocalized in the ER (Sullivan et al., 1999). Expression of some mutant forms of lamin A from patients with autosomal dominant Emery-Dreifuss in transfected cells causes a loss of some emerin from the nuclear envelope (Raharjo et al., 2001; Östlund et al., 2001). Hence, mutations in an integral inner nuclear membrane protein and associated lamins may lead to an overall disruption in the structural integrity of the nuclear envelope, causing increased lateral mobility of integral proteins in the inner nuclear membrane. Measurements of the diffusional mobilities of integral proteins of the inner nuclear membrane in cells from patients with Emery-Dreifuss muscular dystrophy and Dunnigan-type partial lipodystrophy may provide further insights into how mutations in emerin and lamins A and C lead to alterations in the inner nuclear membrane in inherited human diseases. Increased lateral diffusion of integral proteins in the inner nuclear membrane and possibly back into the contiguous ER may lead to structural weakness in cells and cause tissue-specific defects that result in skeletal muscular dystrophy and cardiomyopathy.

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