Intracellular trafficking of emerin, the Emery-Dreifuss muscular dystrophy protein

Cecilia Östlund1, Jan Ellenberg2, Einar Hallberg3, Jennifer Lippincott-Schwartz2 and Howard J. Worman1,*

1Departments of Medicine and of Anatomy and Cell Biology, College of Physicians and Surgeons, Columbia University, New York, New York 10032, USA
2Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892, USA
3Center for Biotechnology, NOVUM, Karolinska Institute, 141 57 Huddinge, Sweden
*Author for correspondence (E-mail: hjw14@columbia.edu)

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SUMMARY
Emerin is an integral protein of the inner nuclear membrane that is mutated or not expressed in patients with Emery-Dreifuss muscular dystrophy. Confocal immunofluorescence microscopy studies of the intracellular targeting of truncated forms of emerin, some of which are found in patients with Emery-Dreifuss muscular dystrophy, show that the nucleoplasmic, amino-terminal domain is necessary and sufficient for nuclear retention. When this domain is fused to a transmembrane segment of an integral membrane protein of the ER/plasma membrane, the chimeric protein is localized in the inner nuclear membrane. The transmembrane segment of emerin is not targeted to the inner nuclear membrane. Fluorescence photobleaching experiments of emerin fused to green fluorescent protein demonstrate that the diffusional mobility (D) of emerin is decreased in the inner nuclear membrane (D=0.10±0.01 μm²/second) compared to the ER membrane (D=0.32±0.01 μm²/second). This is in agreement with a model where integral proteins reach the inner nuclear membrane by lateral diffusion and are retained there by association with nucleoplasmic components. Some overexpressed emerin-green fluorescent protein also reaches the plasma membrane of transfected cells, where its diffusion is similar to that in the inner nuclear membrane, suggesting that emerin may also associate with non-nuclear structures.

Key words: Nuclear envelope, Membrane protein, Protein targeting, Fluorescence photobleaching, Muscle disease

INTRODUCTION
Emery-Dreifuss muscular dystrophy (EDMD; OMIM # 310300) is an X-linked disease characterized by early contractures of the elbows, Achilles’ tendons and posterior neck, slow progressive muscle wasting and cardiomyopathy with atrioventricular conduction block (Emery and Dreifuss, 1966; Rowland et al., 1979; Emery, 1989). Positional cloning has identified a gene on chromosome Xq28 that is mutated in individuals with EDMD (Bione et al., 1994). The protein encoded by this gene, called emerin, is a type II integral membrane protein of 254 amino acids, with a 219 amino acid amino-terminal domain followed by a 21 amino acid transmembrane segment 11 residues from the carboxyl terminus (Bione et al., 1994). Emerin, which when mutated or absent is associated with a skeletal- and heart-muscle specific phenotype, is expressed in the inner nuclear membrane of virtually all somatic cells and absent from this location in most individuals with EDMD (Manilal et al., 1996; Nagano et al., 1996). A phenotypically similar disorder(s) with cardiac condition abnormalities as a prominent feature is inherited in an autosomal dominant manner (Miller et al., 1985; Gilchrist and Leshner, 1986; Emery, 1989). Recently, mutations in the Lamin A/C gene have been found in individuals with this form of EDMD (Bonne et al., 1999).

The inner nuclear membrane is one of three distinct membrane domains of the interphase nuclear envelope. On its nucleoplasmic face it is associated with the nuclear lamina, an intermediate filament meshwork composed of proteins called lamins (Aebi et al., 1986; Fisher et al., 1986; McKeon et al., 1986). Five integral membrane proteins, some with multiple isoforms generated by alternative RNA splicing, have so far been identified as specifically localized to the inner nuclear membrane. These five proteins are emerin (Manilal et al., 1996; Nagano et al., 1996), lamin B receptor (LBR) (Worman et al., 1988, 1990; Ye and Worman, 1994), lamina associated polypeptide (LAP)1 (Senior and Gerace, 1988; Martin et al., 1995), LAP2/thymopoietin (Foisner and Gerace, 1993; Harris et al., 1994; Furukawa et al., 1995) and MAN1 (F. Lin, D. L. Blake, I. Callebaut, I. S. Skerjanc, M. W. McBurney, M. Paulin-Levasseur and H. J. Worman, unpublished). The precise functions of these proteins remain to be determined, but LBR, LAP1 isoform C and LAP2/thymopoietin-β have been shown to bind lamins and...
LBR and LAP2/thymopoietin-β have also been shown to bind chromatin (Worman et al., 1988; Foisner and Gerace, 1993; Ye and Worman, 1994, 1996; Ye et al., 1997). LBR also shows homology with sterol reductases (Schuler et al., 1994; Holmer et al., 1998).

Only a few studies have addressed how integral proteins are targeted to the various nuclear envelope membrane domains (Wozniak et al., 1992; Smith and Blobel, 1993; Soullam and Worman, 1993, 1995; Furukawa et al., 1995; Ellenberg et al., 1997; Söderqvist et al., 1997; Cartegni et al., 1997; Ellis et al., 1998). In contrast, there has been major progress in understanding the targeting of soluble proteins to the nucleus in recent years (for reviews see Görlich and Mattaj, 1996; Pemberton et al., 1998). Signals capable of targeting soluble proteins through the nuclear pores cannot target integral membrane proteins to the inner nuclear membrane (Soullam and Worman, 1995). These proteins likely reach their final localization in the inner nuclear membrane by lateral diffusion in the endoplasmic reticulum (ER), outer nuclear and pore membranes after synthesis on the ER (Soullam and Worman, 1993, 1995; Furukawa et al., 1995; Ellenberg et al., 1997). According to this model, integral proteins are then retained in the inner nuclear membrane by binding to structures in the nucleus, such as the lamina, chromatin or other nuclear proteins. In vivo studies of LBR fused to green fluorescent protein (GFP) have shown that LBR is relatively immobile in the nuclear envelope, while it diffuses more freely in the ER, supporting this diffusion/retention model (Ellenberg et al., 1997).

The molecular mechanism by which aberrant or no expression of the ubiquitously expressed inner nuclear membrane protein emerin can cause muscular dystrophy is not understood. Cartegni et al. (1997) reported the localization of a protein recognized by anti-emerin antibodies to desmosomes and fasciae adherentes of intercalated discs, which are situated at the site of attachment of cardiac muscle cells to their neighbors, and suggested that this accounts for the heart abnormalities in patients with EDMD. These data were recently contradicted in a study by Manilal et al. (1999), where antibodies did not recognize the intercalated discs after affinity purification. Even if emerin is present in the intercalated discs it does not, however, explain the skeletal muscle abnormalities in EDMD. Furthermore, it is not clear how emerin can reach two distinct intracellular locations in cardiac muscle, the inner nuclear membrane and the intercalated discs. We therefore decided to investigate the intracellular trafficking of emerin and how it is affected in mutant forms of the protein.

**MATERIALS AND METHODS**

**Plasmid construction**

Constructs that expressed FLAG-tagged polypeptides were made in plasmid pSVK3 (Pharmacia Biotech, Inc., Piscataway, NJ), which contains a multiple cloning site downstream from the SV-40 early promoter. All cloning procedures were performed according to standard methods (Sambrook et al., 1989). Nucleotide numbering of emerin is based on its previously reported sequence (Bione et al., 1995). cDNAs for cloning were generated by polymerase chain reaction (PCR) (Saiki et al., 1987), using the Gene Amp PCR System 2400 (Perkin-Elmer Cetus Corp., Norwalk, CT), with restriction sites engineered at the 5’ ends of the primers. A human emerin cDNA clone obtained from American Type Culture Collection (Manassas, VA) was used as a template. In most instances, a SmaI site was engineered within the sense PCR primer and a Clal site in the antisense PCR primer. PCR products were digested with these restriction endonucleases and ligated into plasmid pBFT4, which allowed digestion with SmaI and Clal. pBFT4 is a Bluescript II KS (Stratagene, La Jolla, CA)-based plasmid containing a Kozak sequence, ATG and the FLAG-tag coding sequence 5’ to the multiple cloning site. The resulting plasmids, which contained an Spel site 5’ to the Kozak sequence and an Xhol site 3’ to the cloned insert, were then digested with Spel and Xhol and the isolated cassette ligated into pSVK3 that was digested with XbaI (an isoschizomer of SpeI) and Xhol.

To construct the plasmid expressing an emerin-chicken hepatic lectin (CHL) fusion protein, cDNA encoding a FLAG-tag followed by amino acids (aa) 3-219 of emerin was ligated into pSVK3 as described above, using an antisense primer containing both a BspEI site and a Clal site for PCR amplification. This plasmid was digested with BspEI and Xhol and ligated with cDNA encoding aa 24-131 of CHL isolated from plasmid LMBR-CHL (Soullam and Worman, 1993).

To express a protein with aa 117-170 of emerin fused to CHL, a cDNA cassette isolated from plasmid LMBR-CHL (Soullam and Worman, 1993) by digestion with EcoRI and Xhol was ligated into the EcoRI and Xhol sites of pBFT4. The ligation product was then digested with SmaI (which has a site in the pBFT4 multiple cloning site), and BspEI (which has a site that is 117-170 of emerin, amplified using a sense primer with a SmaI site and an antisense primer with a BspEI site, was ligated into the CHL-containing vector, yielding a cDNA encoding a FLAG-tagged chimeric protein with aa 117-170 of emerin fused to aa 24-131 of CHL. This cDNA cassette was isolated by digestion with Spel and Xhol and ligated into pSVK3 as described above.

For construction of emerin-chicken muscle pyruvate kinase (CMPK), a cDNA fragment encoding aa 17-476 of CMPK was isolated from plasmid p3PK (Frangioni and Neel, 1993) by digestion with HindIII and Clal. This fragment was ligated into pBFT4 that was digested with HindIII and Clal. The resulting plasmid was then digested with BgII and SmaI and a PCR product encoding aa 3-219 of emerin, generated using a sense primer with an engineered SmaI site and an antisense primer with an engineered BamHI site, was ligated into it. The resulting chimeric cDNA was then subcloned into pSVK3 as described above.

For construction 3-169(208), PCR amplification was performed using a sense primer containing a SmaI site. The antisense primer encoded a modified stretch of emerin including a two-base-pair deletion at nucleotides 1564 and 1565 found in patient LB1520 described by Bione et al. (1994) and a SacI site found in the emerin gene at nucleotide 1580. The PCR product was digested with SmaI and SacI and ligated into the SmaI and SacI sites of plasmid pACT2 (CLONTECH Laboratories, Inc., Palo Alto, CA). The resulting plasmid, encoding a mutated form of the first 174 aa of emerin, was then digested with SacI and Xhol and ligated with wild-type emerin cDNA encoding aa 175-254, isolated from full-length emerin cDNA in pBFT4 digested with SacI and Xhol. The ligation product, encoding emerin with nucleotides 1564 and 1565 deleted, was digested with SmaI and Xhol and ligated into pBFT4 digested with SmaI and Xhol, and then ligated into pSVK3 as above.

For studies of emerin fused to GFP, a FLAG-tagged construct encoding aa 3-254 of emerin was fused, via its carboxyl terminus, to the P64L, S65T, H231L variant of GFP using the expression vector pEGFP-N1 (CLONTECH Laboratories, Inc.). A PCR product encoding aa 117-170 of emerin, amplified using a sense primer with an engineered SmaI site and an antisense primer with an engineered SmaI site, was ligated into the plasmid pACT2, yielding a cDNA encoding a FLAG-tagged chimeric protein with aa 117-170 of emerin fused to aa 24-131 of CHL. This cDNA cassette was isolated by digestion with Spel and Xhol and ligated into pSVK3 as described above.

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then digested with SacI and EcoRI and ligated with cDNA encoding the FLAG-tag fused to aa 3-63 of emerin, that was isolated from emerin-pBFT4 digested with SacI and EcoRI.

**Cell culture and transfection**

COS-7 cells were grown in minimal essential medium containing 10% fetal bovine serum (Life Technologies, Gaithersburg, MD). Cells were transfected using DMRIE-C (Life Technologies), following the manufacturer’s instructions. The cells were overlaid with the lipid-DNA complexes for 10-22 hours. When transfecting cells with pSVK3-based constructs, the cells were allowed to grow in fresh medium for approximately 36 hours post-transfection. Cell cultures were then split into chamber slides and grown for an additional 12-24 hours before preparation for immunofluorescence microscopy. For studies of emerin fused to GFP, cells were transfected directly on the chamber slides and prepared for microscopy 6-24 hours after transfection. For brefeldin A treatment, cells transfected as above with FLAG-tagged emerin or FLAG-tagged emerin fused to GFP were incubated in medium containing 2 μg/ml brefeldin A (Epiconcentres Technologies, Madison, WI) for 2.5, 4, 6, 8 or 10 hours. Brefeldin A was added when the lipid-DNA complex was removed, a time point when there still was little or no expression of emerin-GFP. The cells were then examined by immunofluorescence microscopy using the anti-FLAG M5 antibody as described below.

**Immunofluorescence microscopy**

Transfected cells were washed three times with phosphate-buffered saline (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 (BSA) (solution B) for 1 hour at 37°C. The primary antibodies used were anti-FLAG M2 monoclonal antibody or anti-FLAG M5 monoclonal antibody at a dilution of 1:200 (Sigma, St. Louis, MO), anti-lamin B1 polyclonal antibody (Cance et al., 1992) at a dilution of 1:2,000 or anti-GM130 polyclonal antibody (Nakamura et al., 1995) at a dilution of 1:200. After four washes with solution A, the cells were incubated with the secondary antibodies diluted 1:200 as described for the primary antibodies. 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 lissamine-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 coverslips mounted using anti-fade mounting medium (5% propyl gallate, 0.25% 1,4-diazabicyclo-(2,2,2)-octane, 0.0025% phenylenediamine in glycerol). For digitonin-permeabilization, the 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 precooled 40 μg/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 on 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 (FRAP) of live emerin-GFP expressing cells was performed on a 37°C stage of a Zeiss LSM 410 (Carl Zeiss, Inc.) essentially as described (Ellenberg et al., 1997). Briefly, for qualitative experiments shown in Fig. 6, the outlined box was photobleached with a high zoom 488 nm laser scan using the full output of a 75 mw Ar/Kr laser. Immediately afterwards, recovery was monitored at 9-second intervals with attenuated laser emission (1/100 to 3/1000 of the bleach intensity) at low zoom, without further photobleaching during the course of the experiment. To determine the effective diffusion constant, D, additional quantitative FRAP experiments were performed. Here a 4 μm wide strip that extended across the cell and through its entire depth was photobleached at full laser power and high zoom and recovery was followed only within the photobleached strip with attenuated emission (1/1000 of the bleach intensity) and low zoom at 3-second intervals without further photobleaching of the sample. D was determined by fitting the experimental data (Fig. 7) to an empirical formula for one-dimensional diffusion (Ellenberg et al., 1997) and verified by a numerical computer simulation of free diffusion on the qualitative and quantitative data sets (Sciky et al., 1997). Fluorescence loss in photobleaching (FLIP; Cole et al., 1996) of emerin-GFP expressing cells (shown in Fig. 8) used cycles of photobleaching of the indicated areas at full laser illumination and a single whole-cell image with attenuated emission (9/1000 of the bleach intensity) to monitor loss of fluorescence outside the photobleached area. This routine was repeated every 80 seconds until ER fluorescence was completely lost to probe the continuity of ER and nuclear envelope pools of emerin-GFP.

**Other chemicals**

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

**RESULTS**

**The amino-terminal domain of emerin is necessary and sufficient for inner nuclear membrane targeting and retention**

To determine the domain of emerin responsible for its inner nuclear membrane localization, we transiently transfected COS-7 cells with plasmids expressing full-length emerin and truncated forms of the protein. For detection of the expressed proteins, nucleotide sequences encoding FLAG-epitopes were fused to the 5’-ends of the constructs (Fig. 1A). Full-length emerin was localized to the nuclear rim, showing colocalization with lamin B1, a marker for the lamina/nuclear envelope (Fig. 1B). A truncated emerin (residues 3-219), consisting of the amino-terminal nucleoplasmic domain but lacking the transmembrane segment, was retained within the nucleus. This truncated emerin did not give a nuclear rim staining but rather a diffuse labeling of the nucleus (Fig. 1B). The carboxyl-terminal part of emerin (residues 197-254) containing the transmembrane segment and the luminal tail did not accumulate at the nuclear rim but was localized to the entire nucleus. This truncated emerin did not give a nuclear rim staining but rather a diffuse labeling of the nucleus (Fig. 1B). The hydrophobic region of emerin from aa 223-243 is a functional transmembrane segment, but that it is not retained in the inner nuclear membrane.

To confirm that the FLAG-tagged, full-length emerin construct localized to the inner nuclear membrane, as opposed to the outer nuclear membrane, the plasma membranes of cells expressing this construct were selectively permeabilized with
Most cells showed no staining with the anti-FLAG antibodies; some overexpressing cells showing a typical ER-staining with no defined nuclear rim-staining (data not shown). To further confirm that the nuclear envelope was intact and FLAG-tagged emerin in the inner nuclear membrane was not accessible to anti-FLAG antibodies in digitonin-permeabilized cells, cells expressing FLAG-tagged emerin fused to GFP were treated with Triton X-100 or digitonin (Fig. 2). The endogenous fluorescence from GFP was then visualized simultaneously to antibody labeling with anti-FLAG antibodies recognized by FITC-conjugated secondary antibodies (left) and polyclonal antibodies against lamin B1 recognized by rhodamine-conjugated secondary antibodies (middle). 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, the nuclear rim was detected by GFP fluorescence but was not labeled with anti-FLAG antibodies. This experiment clearly demonstrated that the majority of emerin-GFP was located in the inner nuclear membrane.

To determine if the amino-terminal domain of emerin was able to target other transmembrane polypeptides to the inner nuclear membrane, COS-7 cells were transfected with a plasmid encoding a chimeric protein consisting of the amino-terminal domain of emerin fused to the transmembrane segment and a portion of the luminal region of CHL (Fig. 3A). CHL is a type II integral membrane protein of the ER, endosomes and plasma membrane (Chiacchia and Drickamer, 1984; Mellow et al., 1988). It has a cytoplasmic, amino-terminal domain of 23 amino acids followed by a transmembrane domain and a luminal, carboxyl-terminal domain of 160 amino acids. The emerin-CHL fusion protein localized to the nuclear rim, similar to full-length emerin (Fig. 3B). That the fusion protein was localized to the inner nuclear membrane was confirmed with immunofluorescence of cells permeabilized with digitonin (data not shown). To determine if the amino-terminal domain of emerin could also target a non-membrane, cytosolic protein to the nucleus, this domain was fused to a truncated version of CMPK, which is a soluble, cytosolic protein (Frangioni and Neel, 1993) (Fig. 3A). This fusion protein, of approximately 75 kDa, contrary to the amino-terminal domain of emerin itself, is too large to diffuse freely into the nucleus (Paine et al., 1975). When expressed in

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**Fig. 1.** The amino-terminal domain of emerin contains a nuclear envelope targeting signal. (A) Schematic diagrams of proteins expressed by the plasmid constructs used in experiments in which COS-7 cells were transfected. Domains of the proteins are represented by: black, FLAG-tag; white, nucleoplasmic and luminal domains of emerin; gray, transmembrane segment of emerin. The amino terminus of each protein is at the left. (B) Cellular localization of full-length emerin (aa 3-254), the amino-terminal domain (aa 3-219) and the carboxyl-terminal domain (aa 197-254) as determined by laser scanning confocal immunofluorescence microscopy. Antibodies used were anti-FLAG monoclonal antibodies recognized by FITC-conjugated secondary antibodies (left) and polyclonal antibodies against lamin B1 recognized by rhodamine-conjugated secondary antibodies (middle). The pictures to the right show an overlay of the FITC and rhodamine channels. Bars, 10 μm.
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COS-7 cells, the emerin-CMPK fusion protein remained in the cytosol and was excluded from the nucleus (Fig. 3B). In conclusion, the emerin amino-terminal domain is capable of localizing an integral membrane protein to the inner nuclear membrane but not a soluble protein larger than 60 kDa to the nucleus.

Fig. 2. Emerin localizes to the inner nuclear membrane. (A) Schematic diagrams of the protein expressed by the transfected plasmid construct. Domains of the protein are represented by: black, FLAG-tag; white, nucleoplasmic and lumenal domains of emerin; gray, transmembrane segment of emerin; hatched, GFP. (B) Immunofluorescence studies of COS-7 cells that were transfected with plasmids expressing emerin tagged with a FLAG-tag and fused to GFP. The cells were permeabilized with Triton X-100 (upper panels) or digitonin (lower panels) and were then labeled with anti-FLAG monoclonal antibodies recognized by rhodamine-conjugated secondary antibodies (left panels). The middle panels show fluorescence from GFP and the panels to the right an overlay of signals from the two channels. Bar, 10 μm.

Fig. 3. The targeting signal/signals in the amino-terminal domain of emerin can direct an unrelated integral membrane protein to the inner nuclear membrane but not a non-membrane protein to the nucleoplasm. (A) Schematic diagrams of proteins expressed by the plasmid constructs used in transfection experiments. Domains of the proteins are represented by: black, FLAG-tag; white, nucleoplasmic domain of emerin; hatched, CHL; gray, CMPK. (B) Immunofluorescence images from laser scanning confocal immunofluorescence microscopy showing localization of the amino-terminal domain of emerin fused to truncated CHL (upper panels) and the amino-terminal domain of emerin fused to truncated CMPK (lower panels). Antibodies used were anti-FLAG monoclonal antibodies recognized by FITC-conjugated secondary antibodies (left) and polyclonal antibodies against lamin B1 recognized by rhodamine-conjugated secondary antibodies (middle). The pictures to the right show an overlay of the FITC and rhodamine channels. Bars, 10 μm.
Intracellular distribution of mutant forms of emerin found in patients with EDMD

More than 70 different mutations in the emerin gene have been identified in patients with EDMD (Yates, 1997; http://www.path.cam.ac.uk/emd/mutation.html). To narrow down the region responsible for targeting of emerin, the intracellular localizations of some of these mutated forms were studied (Fig. 4). Two short, truncated forms of emerin, aa 3-41 and 3-44, were apparently unstable and virtually impossible to detect in cells by immunofluorescence microscopy (data not shown). Truncated forms of emerin, aa 3-74 and 3-109, showed a diffuse staining of the entire cell. These proteins are small enough to diffuse freely through the nuclear pores (Paine et al., 1975), but seem to lack the signal responsible for nuclear retention present in the full emerin amino-terminal domain. When a truncated emerin containing aa 3-147 was expressed, there was a clear accumulation in the nucleoplasm but also significant staining of the cytoplasm. A nuclear rim staining pattern was seen with a frame-shift mutation at aa 169 (3-169(208)). This construct lacks the native transmembrane domain of emerin, as the frame shift generates a stop codon at aa 208. The out-of-frame region coincidentally encodes a completely unrelated hydrophobic stretch of amino acids (Cartegni et al., 1997). Digitonin-permeabilization confirmed the protein to be localized to the inner nuclear membrane (data not shown). Truncated forms of emerin, aa 3-205 and 3-228, showed a clear accumulation in the nucleus; however, not at the nuclear periphery but in a diffuse nuclear distribution as would be expected for proteins lacking a membrane-spanning segment.

aa 117-170 in the amino-terminal domain of emerin is retained in the nucleus but is not sufficient to target an integral membrane protein to the inner nuclear membrane

The results of the above experiments suggested that the nuclear retention signal/signals of emerin can be limited to the region between aa 109 and 169. To verify this, additional constructs, encoding aa 3-170, 170-254 and 117-170 were made (Fig. 5A). When COS-7 cells were transfected with these constructs, immunofluorescence showed a nuclear localization of the polypeptides from aa 3-170 and 117-170 (Fig. 5B). This showed that these regions can retain a non-membrane protein in the nucleoplasm. The polypeptide from aa 170-254 was localized to the entire ER (Fig. 5B), providing further evidence for the lack of inner nuclear membrane retention signals in the carboxyl-terminal region of emerin. When aa 117-170 were fused to CHL (Fig. 5A), the polypeptide remained in the ER and did not accumulate in the inner nuclear membrane (Fig. 5B). The retention signal between aa 117-170 of emerin, which is adequate for retention of a non-membrane protein in the nucleus, is therefore not sufficient for retention of an integral protein in the inner nuclear membrane. Efficient inner nuclear membrane retention required the aa 3-170 portion of emerin.

Lateral diffusion of emerin in the ER, inner nuclear membrane and plasma membrane

To determine if emerin becomes immobilized when it reaches the inner nuclear membrane, photobleaching experiments were performed on emerin with GFP fused to its carboxyl terminus. FRAP was performed on emerin-GFP localized to the nuclear envelope (Fig. 6A). In many cells emerin-GFP localized partly to the ER. This staining was predominantly seen in cells overexpressing emerin-GFP at a relatively high level, and it is
likely that it represents proteins ‘backed up’ in the ER when its binding/retention sites in the nuclear membrane are filled. A quantitative analysis of the correlation between expression levels and ER staining was not possible as the population of cells showed a high variability in the degree of ER staining. FRAP performed on emerin-GFP localized to the ER is shown in Fig. 6B. In many cells overexpressing emerin-GFP, the protein was also present in the Golgi apparatus, colocalizing with the cis-Golgi matrix protein GM130 (data not shown; Nakamura et al., 1995). Transport of emerin through the Golgi apparatus is, however, not necessary for its localization to the inner nuclear membrane as transfected cells treated with brefeldin A still showed an inner nuclear membrane localization of emerin-GFP (data not shown). Brefeldin A blocks protein transport from the ER to the Golgi apparatus and causes the Golgi apparatus to disassemble (Misumi et al., 1986; Lippincott-Schwartz et al., 1989; Sciaky et al., 1997).

When emerin-GFP was analyzed by FRAP, the membrane pools of emerin in the nuclear envelope and ER exhibited different mobilities. The bleached nuclear envelope area did not regain full fluorescence intensity 260 seconds after photobleaching (Fig. 6A). After approximately 8 minutes, the fluorescence recovery was nearly complete (data not shown). In the bleached ER area, fluorescence recovered almost immediately and regained most of its original fluorescence intensity after approximately 60 seconds (Fig. 6B). When cells overexpressing emerin-GFP were examined 36 hours after transfection, another distinct pool of protein could be distinguished in the plasma membrane (Fig. 6C). When analyzed by FRAP, the recovery rate in the plasma membrane was similar to that in the nuclear envelope and distinctly slower compared to the recovery rate in the ER.

Quantitative FRAP experiments were performed to determine the diffusion constants \( D \) of the different membrane pools (Fig. 7). \( D \) was 0.32±0.01 \( \mu m^2/\text{second} \) for the ER pool, 0.10±0.01 \( \mu m^2/\text{second} \) for the nuclear envelope pool and 0.09±0.02 \( \mu m^2/\text{second} \) for the plasma membrane pool (Table 1). Emerin was therefore significantly less mobile in both the inner nuclear membrane and plasma membrane relative to the ER.

We also used FLIP to examine emerin-GFP diffusion. In FLIP, an area of the cell is repeatedly photobleached and the fluorescence loss in the entire cell is measured. When parts of the ER of cells expressing emerin-GFP at high levels were repeatedly bleached, the nuclear envelope retained its fluorescence after all fluorescence had disappeared from the ER (Fig. 8). The Golgi apparatus also retained its fluorescence during the experiment. These results show that the diffusional capacity of emerin decreases when the protein enters the inner nuclear membrane. Although there is a slow lateral movement of emerin within the inner nuclear membrane, there is very little back flow of protein from the inner nuclear membrane to the ER. There is also very little flow of emerin-GFP from the Golgi back to the ER.

### DISCUSSION

We have shown that the nucleoplasmic, amino-terminal domain of emerin is necessary and sufficient for targeting to and retention in the inner nuclear membrane while the transmembrane segment and luminal carboxyl-terminal domain localizes to the ER when expressed by itself. This finding is in disagreement with another study, which claimed that the transmembrane segment of emerin was the main determinant for inner nuclear membrane targeting (Cartegni et al., 1997). Our results show that while the transmembrane...
segment gets inserted into the ER/outer nuclear envelope, it is unable to retain the protein in the inner nuclear membrane. Our results using FRAP and FLIP also show that the targeting of emerin to the inner nuclear membrane is consistent with the ‘diffusion and retention’ model proposed based on studies of LBR (Soullam and Worman, 1995; Ellenberg et al., 1997). In this model, proteins synthesized on the ER can diffuse to the inner nuclear membrane and are retained there by binding to nuclear ligands such as lamins or chromatin.

Most of the integral proteins of the inner nuclear membrane and the nuclear pore membrane, for example LBR, LAP2, POM121 and gp210, contain targeting and retention signals in the parts of the proteins directed towards the nucleocytoplasmic side of the nuclear membrane (Wozniak and Blobel, 1992; Soullam and Worman, 1993, 1995; Furukawa et al., 1995; Söderqvist et al., 1997). These domains likely contain regions that bind to nuclear or pore complex proteins (or possibly nucleic acids). There are also examples where the hydrophobic transmembrane segments contribute to inner nuclear membrane retention, for example in gp210 and LBR.

**Fig. 6.** The mobility of emerin is decreased in the nuclear envelope and the plasma membrane compared to the ER. Confocal fluorescence studies of localization and mobilities of emerin-GFP in interphase cells by qualitative FRAP-experiments on cells expressing emerin fused to GFP, showing photobleach recovery in (A) nuclear envelope membranes, (B) ER membranes and (C) the plasma membrane. The fluorescence in the boxed regions was bleached and the fluorescence recovery is shown at 9 seconds (9s), 60 seconds (60s) and 260 seconds (260s) after the bleaching. Bars, 5 μm.
Intracellular trafficking of emerin (Wozniak and Blobel, 1992; Smith and Blobel, 1993; Soullam and Worman, 1995). These transmembrane segments may contribute to retention by homodimerization within the plane of the membrane. It has previously been shown that the signals retaining integral proteins in the inner nuclear membrane differ from the nuclear localization signals, which target soluble proteins through the nuclear pores (Soullam and Worman, 1995). Our results show that the nucleoplasmic domain of emerin, like the nucleoplasmic domain of LBR, is able to target CHL to the nuclear membrane. The emerin amino-terminal domain is, however, unable to target a cytosolic protein greater than 60 kDa to the nucleus, which lends further weight to the hypothesis that the mechanisms for nuclear targeting differ between soluble and integral membrane proteins.

The amino-terminal domain of emerin likely contains at least two, separate signals for inner nuclear membrane retention. One may be localized between aa 117 and 170, as a non-membrane construct containing only this stretch of emerin is retained in the nucleus. It does not, however, contain sufficient information to target the truncated form of CHL, implying the presence of an additional signal important for inner nuclear membrane targeting in another part of the amino-terminal domain. One of the mutated forms of emerin identified in a patient with EDMD has a mutation at aa 169, which causes a frame shift, yielding a region translated out-of-frame between aa 169 and 208. This mutant form of emerin, as previously described by Cartegni et al. (1997) and demonstrated in the present study, localizes to the inner nuclear membrane. The out-of-frame region of this mutant form of emerin coincidentally encodes a hydrophobic stretch of amino acids, completely unrelated to the wild-type transmembrane region of emerin. It is difficult to believe, as proposed by Cartegni et al. (1997), that this fortuitously encoded transmembrane segment can be an inner nuclear membrane targeting signal. More likely, the portion of the amino-terminal domain of emerin from aa 1-169 is sufficient for nuclear retention of a transmembrane protein. These results suggest that the second necessary, but not sufficient, inner nuclear membrane retention signal must exist in emerin at the amino-terminal side of aa 117.

Emerin shows two regions of homology with LAP2/thymopoietin-β, one of the lamin-binding proteins of the inner nuclear envelope (Bione et al., 1994; Furukawa et al., 1995). The first of these regions, between aa 6-44 of emerin and aa 114-152 of LAP2, is also conserved in another inner nuclear membrane protein, MAN1 (F. Lin, D. L. Blake, I. Callebaut, I. S. Skerjanc, M. W. McBurney, M. Paulin-Levasseur and H. J. Worman, unpublished). The second of these regions is between aa 221-254 of emerin and 409-442 of LAP2. These regions of homology are not in domains implicated in lamin-binding and nuclear envelope targeting (aa 298-373) or chromosome interaction (aa 1-85) of LAP2 (Furukawa et al., 1995, 1998). Therefore, emerin and LAP2 do not appear to be retained in the inner nuclear membrane by
conserved signals. Some studies have suggested that emerin may also interact with nuclear lamins (Cartegni et al., 1997; Ellis et al., 1998; Squarzoni et al., 1998), suggesting that a different lamin binding domain may be important in the inner nuclear membrane retention of the protein.

Using FRAP and FLIP, we have shown that emerin has a decreased lateral mobility in the inner nuclear membrane compared to the ER. Interestingly, the diffusion coefficient of emerin in the inner nuclear membrane is not as low as that for LBR (Ellenberg et al., 1997). Emerin therefore is not completely immobilized in the inner nuclear membrane by an irreversible binding to an immobile nuclear structure, for example chromatin, which is immobile in the interphase nucleus (Abney et al., 1997; Marshall et al., 1997). Emerin may instead bind to non-chromatin nuclear proteins (or nucleic acids) and diffuse more slowly as a complex in the inner nuclear membrane than in the ER. This can result in a steady state concentration significantly greater in the inner nuclear membrane than in the continuous ER in interphase cells. This is consistent with the FRAP experiments showing decreased diffusion in the inner nuclear membrane and the FLIP experiments showing little exchange between the inner nuclear membrane and the ER. A large nuclear protein complex would be expected to exhibit a slow lateral diffusion in the inner nuclear membrane and to be unable to diffuse back to the ER through the nuclear pore lateral channels.

How can mutations in emerin, which is expressed in virtually all cells, lead to abnormalities that are specific to heart and certain skeletal muscles? One possibility is that nuclear envelope proteins such as emerin interact with chromatin proteins, transcription factors or DNA sequences that are involved in regulating gene expression in cardiac and skeletal muscle cells. For example, inner nuclear membrane protein LBR binds to HP1 chromatin proteins (Ye and Worman, 1996; Ye et al., 1997) that function in regulating the expression of genes located in or near heterochromatin (Eissenberg et al., 1990). Our data showing that emerin diffuses slowly in the inner nuclear membrane suggests that it is part of a complex that may hypothetically be composed of gene regulatory proteins that are active in muscle cells. Emerin may also be targeted to sites other than the inner nuclear membrane in muscle cells where it plays a role in muscle structure or function. We have shown that at high levels of expression, emerin can reach the plasma membrane where its diffusion is also limited compared to the ER, indicating that emerin may bind to other proteins in a complex at this location. Along these lines, Cartegni et al. (1997) have shown that a protein recognized by antibodies against emerin is localized to the intercalated discs of cardiac myocytes. The absence of, or mutations in, emerin may therefore lead to loss of a function at the plasma membrane of cardiac and skeletal muscle. As the plasma membrane staining was only seen in cells overexpressing emerin-GFP, the physiological relevance of this cellular localization needs to be further studied, as does the localization of emerin to the intercalated discs, which was recently questioned in a study by Manial et al. (1999). Which, if any, of these hypotheses explains how mutations in a nuclear envelope protein can cause muscular dystrophy and cardiomyopathy remains to be established.

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REFERENCES


