

Effect of pathogenic mis-sense mutations in lamin A on its interaction with emerin in vivo

Ian Holt¹, Cecilia Östlund², Colin L. Stewart³, Nguyen thi Man¹, Howard J. Worman² and Glenn E. Morris^{1,*}

¹Biochemistry Group, North East Wales Institute, Wrexham LL11 2AW, UK

²Departments of Medicine and of Anatomy and Cell Biology, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA

³Laboratory of Cancer and Developmental Biology, NCI-FCRDC, PO Box B, Frederick, MD 21702-1201, USA

*Author for correspondence (e-mail: morrisge@newi.ac.uk)

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Summary

Mutations in lamin A/C can cause Emery-Dreifuss muscular dystrophy (EDMD) or a related cardiomyopathy (CMD1A). Using transfection of lamin-A/C-deficient fibroblasts, we have studied the effects of nine pathogenic mutations on the ability of lamin A to assemble normally and to localize emerin normally at the nuclear rim.

Five mutations in the rod domain (L85R, N195K, E358K, M371K and R386K) affected the assembly of the lamina. With the exception of mutant L85R, all rod domain mutants induced the formation of large nucleoplasmic foci in about 10% of all nuclei. The presence of emerin in these foci suggests that the interaction of lamin A with emerin is not directly affected by the rod domain mutations. Three mutations in the tail region, R453W, W520S and R527P, might directly affect emerin binding by disrupting the structure of the putative emerin-binding site, because mutant lamin A localized normally to the nuclear rim but its ability to trap emerin was impaired. Nucleoplasmic foci rarely formed in these three cases (<2%) but, when they

did so, emerin was absent, consistent with a direct effect of the mutations on emerin binding. The lipodystrophy mutation R482Q, which causes a different phenotype and is believed to act through an emerin-independent mechanism, was indistinguishable from wild-type in its localization and its ability to trap emerin at the nuclear rim.

The novel hypothesis suggested by the data is that EDMD/CMD1A mutations in the tail domain of lamin A/C work by direct impairment of emerin interaction, whereas mutations in the rod region cause defective lamina assembly that might or might not impair emerin capture at the nuclear rim. Subtle effects on the function of the lamina-emerin complex in EDMD/CMD1A patients might be responsible for the skeletal and/or cardiac muscle phenotype.

Key words: Nuclear lamina, Mouse knockout, Nuclear envelope, Emery-Dreifuss muscular dystrophy, Lipodystrophy

Introduction

The nuclear envelope consists of two lipid bilayers. The outer membrane is continuous with the endoplasmic reticulum (ER) and is joined to the inner membrane by nuclear pore complexes (NPCs), which mediate molecular trafficking between the cytoplasm and nucleus. The nuclear lamina, a dynamic, fibrous structure located beneath the inner nuclear membrane, is made up of A-type and B-type lamins. Lamins are intermediate filament proteins and have a common structure, consisting of a small N-terminal globular domain, a central helical rod domain of constant length and a globular tail domain of variable size. They enter the nucleus via NPCs and assemble into filaments and thicker fibres, the assembly-disassembly cycle being regulated by lamin phosphorylation. Lamins interact directly with chromatin and several integral membrane proteins, including lamina-associated proteins (LAPs), emerin and the lamin B receptor (Stuurman et al., 1998; Gruenbaum et al., 2001; Hutchison et al., 2001; Morris, 2001; Wilson et al., 2001).

The A-type lamins, A and C, are produced from alternatively spliced mRNA products of the gene on chromosome 1q21.3, lamin C being a shorter form of lamin A (Lin and Worman, 1993). The emerin gene on chromosome Xq28 (Bione et al.,

1994) encodes a 254 amino acid, type II integral membrane protein that is anchored to the inner nuclear membrane by its hydrophobic C-terminal tail (Manilal et al., 1996; Nagano et al., 1996) and interacts directly with lamin A/C (Clements et al., 2000). This interaction involves the globular tail region common to lamins A and C (Vaughan et al., 2001) and a central region (amino acids 70-164) of the emerin molecule (Lee et al., 2001). A similar region of emerin (amino acids 107-175) is required for its localization to the nuclear rim (Tsuchiya et al., 1999; Östlund et al., 1999), suggesting involvement of lamin A/C, and this was confirmed by the location of emerin in the ER in the lamin A/C knockout mouse (Sullivan et al., 1999). However, partial localization of emerin at the nuclear rim was still observed in some knockout mouse tissues, suggesting that factors other than lamin A/C can be involved (Sullivan et al., 1999).

The emerin-lamin A/C interaction is of particular interest because mutations in both proteins cause Emery-Dreifuss muscular dystrophy (EDMD) (Bione et al., 1994; Bonne et al., 1999). In X-linked EDMD, most emerin mutations result in complete absence of emerin protein (Manilal et al., 1998), although reduced emerin levels caused by altered RNA splicing might also result in EDMD (Holt et al., 2001b). A few mis-

sense mutations are known and these produce emerins that are more easily extracted from the nucleus (Fairley et al., 1999). In autosomal dominant EDMD, most mutations are mis-sense and evenly spread throughout the helical rod and globular tail domains common to lamins A and C. It seems likely that these mutations exert a dominant-negative effect on the function of the multisubunit lamin filaments (Morris, 2001). Both forms of EDMD share the same clinical features of early joint contractures, selective muscle wasting and cardiac conduction defects, and they are both extremely variable in severity, suggesting that some specific function of the emerin-lamin A/C complex is affected in both cases (Morris, 2001). Mutations in lamin A/C are also responsible for one form of dilated cardiomyopathy (CMD1A) (Fatkin et al., 1999) and for a limb-girdle muscular dystrophy with conduction defects (type1B) (Muchir et al., 2000). Because these two diseases are closely related to EDMD phenotypically, their molecular pathogenesis is likely to be similar, with variability caused by genetic background (Morris, 2001). In three other diseases, the molecular effects of lamin A/C mutations might be different, because their phenotypes are quite distinct from EDMD. These are Dunnigan-type familial partial lipodystrophy (FPLD) (Cao and Hegele, 2000; Shackleton et al., 2000), autosomal recessive Charcot-Marie-Tooth disorder type 2 (De Sandre-Giovannoli et al., 2002) and inherited mandibuloacral dysplasia (Novelli et al., 2002). In these cases, the mutations might cause gain or loss of specific lamin A/C functions that do not involve the emerin interaction. In FPLD, for example, binding to lamin A/C of a specific transcription factor in adipose tissue might be affected (Lloyd et al., 2002).

Immunohistochemical analysis of wild-type mouse embryonic fibroblasts (MEFs) showed emerin to be concentrated within the nuclear envelope, whereas MEFs from lamin-A-knockout (*lmna*^{-/-}) mice showed a decrease in nuclear-envelope-associated emerin and a more general distribution of emerin in the peripheral ER. Transfection of *lmna*^{-/-} MEFs with human *lamin A* cDNA corrected the localization of emerin to the nuclear envelope. These results suggested that A-type lamin expression is required for the correct localization of emerin (Sullivan et al., 1999). In a more recent study, *lmna*^{-/-} MEFs were transfected with three *lamin A* cDNAs containing pathogenic mis-sense mutations (Raharjo et al., 2001). Wild-type lamin A and lamin A with a lipodystrophy mutation were able to restore the nuclear envelope localization of emerin. However, two lamin A/C mutants causing CMD1A and one causing EDMD were defective in relocating emerin from the peripheral ER to the nuclear rim (Raharjo et al., 2001). A transfection study with a wider range of lamin A/C mutants (Östlund et al., 2001) showed that some EDMD and cardiomyopathy mutants caused the formation of nuclear foci, or aggregates, and disrupted the assembly of endogenous lamins in HeLa and mouse myoblast cell lines.

In the present study, we have advanced these earlier studies by transfecting *lmna*^{-/-} MEFs with a much wider range of lamin A/C mutants, paying particular attention to the effects on the localization of emerin. As a result, we can now rationalize, for the first time, the biological effects of lamin A/C mutations with the known functions of different domains in the lamin A/C molecule. In the new model, EDMD mutations in the globular tail domain affect emerin interaction

directly. Rod mutations affect assembly of the A-type lamina and we hypothesize that any effects on emerin localization at the nuclear rim result indirectly from this.

Materials and Methods

Eukaryotic cell culture and transfection

The cDNAs encoding wild-type lamin A and eight mis-sense mutations were cloned into pSVK3 mammalian expression plasmid (Amersham Pharmacia Biotech) (Östlund et al., 2001). Additionally, cDNA for lamin A (wild-type and R482Q) was cloned into pcDNA4 vector (Invitrogen) (Holt et al., 2001a). Plasmids were purified using EndoFree Plasmid Maxi Kits (Qiagen) and quantified by UV spectrophotometry. The modified pSVK3 vectors add N-terminal FLAG tag to expressed proteins (Östlund et al., 2001) and pcDNA4 vectors add N-terminal Xpress tag.

Eukaryotic cells for transfection were grown in DMEM (Gibco) with 20% decomplexed horse serum (Gibco), 2 mM L-glutamine and antibiotics. Cells used were mouse embryonic fibroblasts from the lamin A null mouse (MEFs *lmna*^{-/-}) (Sullivan et al., 1999), HeLa (human epithelial carcinoma cell line) and COS-7 (African green monkey kidney fibroblast cell line).

Transfection by electroporation was modified from the method of Espinos et al. (Espinos et al., 2001). Briefly, eukaryotic cells were trypsinized and centrifuged, and the cells resuspended to approximately 2×10^6 cells ml⁻¹ in F10-Ham medium (Gibco). 200 μ l cell suspension was mixed with 24 μ g plasmid (or 24 μ g of each plasmid for co-transfections) and placed in an electroporation cuvette with 2 mm gap (BioRad). Cells and plasmid were electroporated at 1.5 kV [resistance = 200 Ω ; capacitance = 25 μ F; time constant (τ) \approx 0.8] using a BioRad Gene Pulser. Following electroporation, the contents of the cuvette were mixed with 1.8 ml of skeletal muscle cell growth medium (PromoCell, Heidelberg) supplemented with 10% decomplexed foetal bovine serum and plated on to four sterile glass coverslips. Cell culture was continued for 48 hours to allow for adherence of cells to the coverslips and expression of transfected plasmid. After the incubation period, cells on coverslips were fixed in 50:50 acetone-methanol for 5 minutes and stored at -80°C .

Immunohistochemistry

Coverslips were brought to room temperature and the cells washed four times with casein buffer (0.1% casein in 154 mM NaCl, 10 mM Tris, pH 7.6). Double labelling of cells was performed by first incubating cells on a coverslip with 50 μ l primary polyclonal antisera for 1 hour at 37°C, washing four times with casein buffer and then incubating with 50 μ l of primary monoclonal antibody (mAb) for 1 hour at 37°C. The anti-emerin antiserum was prepared by immunization of a rabbit with a recombinant human emerin fragment [amino acids 1-188 (Manilal et al., 1996)], which is identical to that used by Raharjo et al. (Raharjo et al., 2001). Primary antibodies, diluted in casein buffer, were as follows. Polyclonals: 1:100 rabbit anti-emerin; 5 μ g ml⁻¹ rabbit anti-FLAG (Sigma, F7425); 1:100 rabbit anti-lamin-B1 (gift of L. Gerace) (Schirmer et al., 2001); 4 μ g ml⁻¹ goat anti-lamin-B (Santa Cruz Biotechnology, sc-6216). Monoclonals: 1 μ g ml⁻¹ anti-LAP2 (Transduction Laboratories, L74520); 20 μ g ml⁻¹ anti-FLAG M2 (Sigma, F3165); 1 μ g ml⁻¹ anti-Xpress (Invitrogen); 1:3 monoclonal antibodies MANEM1 and MANEM8 against emerin (Manilal et al., 1996). Following incubation with primary antibodies, cells were washed four times with casein buffer. 50 μ l per coverslip of secondary antibody pairs (diluted in PBS containing 1% horse serum, 1% foetal bovine serum and 0.1% bovine serum albumin) were then added. The secondary antibody pairs were either 5 μ g ml⁻¹ goat anti-mouse ALEXA 546 and 5 μ g ml⁻¹ goat anti-rabbit ALEXA 488 (Molecular Probes, Eugene, Oregon) or 20 μ g ml⁻¹ horse anti-mouse FITC (Vector Labs, Burlingame, CA) and 50

Table 1. Distribution of transfected lamin A mutants in MEFs (*Imna*^{-/-}) and the ability of transfected lamin A to relocate endogenous emerin from ER to the nuclear rim

| Plasmid/mutation | Associated disease* | Transfection efficiency (%) [†] | Transfected cells that are 'strong transfectants' (%) [‡] | Transfected cells with large nuclear foci (%) [§] | Correctly identified as transfectants (%) ^{**} |
|------------------|---------------------|------------------------------------------|--------------------------------------------------------------------|------------------------------------------------------------|---------------------------------------------------------|
| pSVK3/wild-type | | 38 | 38 | 0 | 72 |
| L85R | CMD1A | 36 | 24 | 1.5 | 42 ^{††} |
| N195K | CMD1A | 24 | 25 | 11.9 [¶] | 42 ^{††} |
| E358K | AD-EDMD | 40 | 30 | 11.1 [¶] | 71 |
| M371K | AD-EDMD | 40 | 25 | 9.8 [¶] | 74 |
| R386K | AD-EDMD | 22 | 29 | 7.6 [¶] | 41 ^{††} |
| R453W | AD-EDMD | 37 | 38 | 1.1 | 49 ^{††} |
| W520S | AD-EDMD | 40 | 46 | 0 | 50 ^{††} |
| R527P | AD-EDMD | 34 | 45 | 2.2 | 45 ^{††} |
| pcDNA4/wild-type | | 49 | 58 | 0 | 95 |
| R482Q | FPLD | 38 | 48 | 0 | 90 |

400 transfected cells from two separate experiments were examined for each plasmid.

*CMD1A, dilated cardiomyopathy; AD-EDMD, autosomal dominant Emery-Dreifuss muscular dystrophy; FPLD, familial partial lipodystrophy.

[†]Percentage of the total cells examined that were transfected.

[‡]Percentage of transfected cells that expressed high levels of transfected lamin A.

[§]Percentage of transfected cells that showed large (>0.7 µm) nuclear foci of transfected lamin A.

[¶]Significantly greater numbers of nuclear foci than wild-type (χ^2 test; $P < 0.01$).

**Values shown were obtained from two separate transfection experiments. For each plasmid, 200 strong transfectants were studied as described in Materials and Methods. The values are the percentage of lamin A transfectants that could be correctly identified from the redistribution of emerin alone.

^{††}Significantly less able to relocate emerin to the nuclear rim compared with appropriate wild-type (χ^2 test, $P < 0.001$).

µg ml⁻¹ rabbit anti-goat TRITC (Sigma, T6028). Secondary antibodies were incubated for 1 hour at 37°C. DAPI (diamidino phenylindole; Sigma), which labels DNA, was added at 200 ng ml⁻¹ in PBS for the final 10 minutes of the incubation to counterstain the nuclei. Cells were then washed four times in casein buffer and mounted in Hydromount (BDH Merck). Cells were examined with a Nikon Eclipse E600 epifluorescence microscope (Nikon UK, Kingston, Surrey, UK) with a 60× objective (numerical aperture 1.40) and a BioRad MicroRadiance 2000 confocal scanning system attachment (BioRad, Hemel Hempstead, UK). Sequential confocal scans were performed with an Argon 488 nm blue excitation laser for green fluorescence from ALEXA 488 and FITC and with a helium/neon 543 nm green excitation laser for red fluorescence from ALEXA 546 and TRITC.

The ability of transfected lamin A to relocate endogenous emerin to the nucleus of MEFs *Imna*^{-/-} was used as an indicator of the degree of direct or indirect interaction of lamin A with emerin. Emerin interaction scoring was performed by two operators. The first operator selected a microscope field that contained between one and five strongly transfected cells and at least 20 non-transfected cells. The filter set was then changed and a second operator attempted to identify which of the cells in the field showed altered distribution of emerin. Altered distribution included a reduction in emerin from the peripheral ER or increased emerin in the nuclear membrane and nucleoplasm, or both. Cells were classed as either 'correctly identified' or 'incorrectly identified'. An alternative approach, in which the operator knew which cell was transfected and had to state whether emerin localization to the nucleus was increased or unaltered, was found to be too subjective, in our hands, to be reliable (see Results).

Results

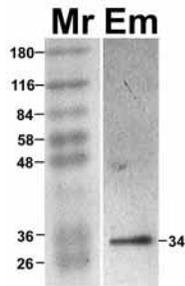
Transfection of lamin A/C-deficient cells with lamin A

Transfection of lamin A/C-deficient MEFs (*Imna*^{-/-}) with a range of lamin A cDNA mutants by electroporation gave an average transfection efficiency of 36% (Table 1). Expression

levels in the transfected cells varied, about one-third showing particularly 'strong' expression of lamin A. A substantial proportion (7.6-11.9%) of cells transfected with four of the mutant lamins (N195K, E358K, M371K and R386K) had large nucleoplasmic foci (Table 1). These results are similar to those reported earlier in HeLa cells and C2C12 mouse myoblasts (Östlund et al., 2001; Raharjo et al., 2001). Any differences from earlier work in the absolute percentage of nuclei with foci might be due to the cell line or culture conditions. In common with an earlier study (Östlund et al., 2001), only foci with diameter greater than 0.7 µm were counted.

In *Imna*^{-/-} MEFs, nearly all emerin is in the ER but lamin A transfection resulted in relocalization to the nucleus (Sullivan et al., 1999). Raharjo et al. (Raharjo et al., 2001) showed that three EDMD/CMD mutations (L85R, N195K and L530P) resulted in loss of ability to relocalize emerin to the nucleus, while the lipodystrophy mutation (R482W) relocalized emerin as well as wild-type lamin A. The main aim of the present study was to extend this work to a wider range of lamin A mutants. Transfected MEFs (*Imna*^{-/-}) were double labelled with a rabbit polyclonal anti-emerin serum and an anti-tag mAb specific for transfected lamin A. Fig. 1 shows that the antiserum is specific for emerin in MEF extracts. Although emerin has homology with LAP2 and MAN1 proteins in their shared LEM domain (reviewed in Morris, 2001), previous studies have also shown that the antiserum does not cross-react with LAP2 (Raharjo et al., 2001). A wide range of changes in the distribution of emerin was observed after transfection with wild-type or mutant lamin A, ranging from emerin remaining in the cytoplasmic ER (Fig. 2B; cf. untransfected Fig. 2A) to emerin being completely relocalized with the transfected lamin A to the nucleus (Fig. 2E). In between, however, were cells with increased nuclear emerin but little apparent decrease in the ER (Fig. 2C,D) and, conversely, cells in which cytoplasmic emerin was decreased with little apparent nuclear change (data not

Fig. 1. Specificity of antiserum for emerin in *Imna*^{-/-} MEFs. MEFs were extracted with a 1% SDS buffer and subjected to SDS-PAGE with prestained molecular weight markers (Sigma), as previously described (Manilal et al., 1996). The western blot was developed with emerin anti-serum (1/1000), peroxidase-labelled pig anti-rabbit Ig (DAKOpatts) and Supersignal chemiluminescent substrate (Pierce). Captured images of prestained blue markers (Mr; lane 1; sizes shown in kDa) and exposed film (Em; lane 2) were aligned after capture. The emerin band at 34 kDa is indicated. There are no higher molecular weight bands of other LEM domain proteins (LAP2 or MAN1). Pre-immune rabbit serum gave no bands at all (not shown).



shown). In many cases, it was difficult to decide whether transfection with lamin A mutants had affected the endogenous emerin or not. Further study might show whether this variability is due to cell cycle differences, mixed cell lineages in MEFs or lamin A expression level.

Effect of pathogenic lamin A mutations on relocalization of emerin to the nuclear rim and on formation of nuclear foci

To quantify the effects of mutations more objectively, we adopted a blind method of assessment, the observer being shown fields of view containing one or several transfected cells (strongly positive for tagged lamin A) plus a four- to 20-fold excess of untransfected cells. The observer was told how many transfected cells were in the field but could not see the lamin A tag fluorescence. Cells correctly identified as transfectants from emerin redistribution (into nucleus, out of cytoplasm or both) were scored as 'hits' (the random hit rate by chance alone would be less than 20%).

Cells transfected with wild-type lamin A in pcDNA4 plasmid were easy to identify from the dramatic changes in emerin distribution and the hit rate of 95% reflects this (Table 1). This hit rate was hardly affected by the R482Q lipodystrophy mutation, confirming earlier results with the R482W mutation (Raharjo et al., 2001). When pSVK3 plasmids were used, it was more difficult to see changes in emerin distribution and the hit rate for wild-type lamin A was only 72%. This might be because the SV40 promoter in pSVK3 gives lower expression levels than the CMV promoter in pcDNA4. The effects of the EDMD and CMD mutations, which were all expressed in the pSVK3 plasmid, are also shown in Table 1. The hit rate was significantly reduced from 72% to 41-50% for six mutants, the L85R and N195K studied previously (Raharjo et al., 2001) and four new mutants, R386K, R453W, W520S and R527P. These mutations seriously impaired the ability to relocate emerin to the nuclear rim but did not appear to abolish it completely. A novel observation from this study is the identification of two rod mutations, E358K and M371K, that cause no loss of ability to relocate emerin (Table 1). Both mutations are near the C-terminal end of the rod domain. All five rod mutants therefore cause visible defects either in lamin A assembly (E358K and M371K) or in emerin capture (L85R) or in both (N195K and

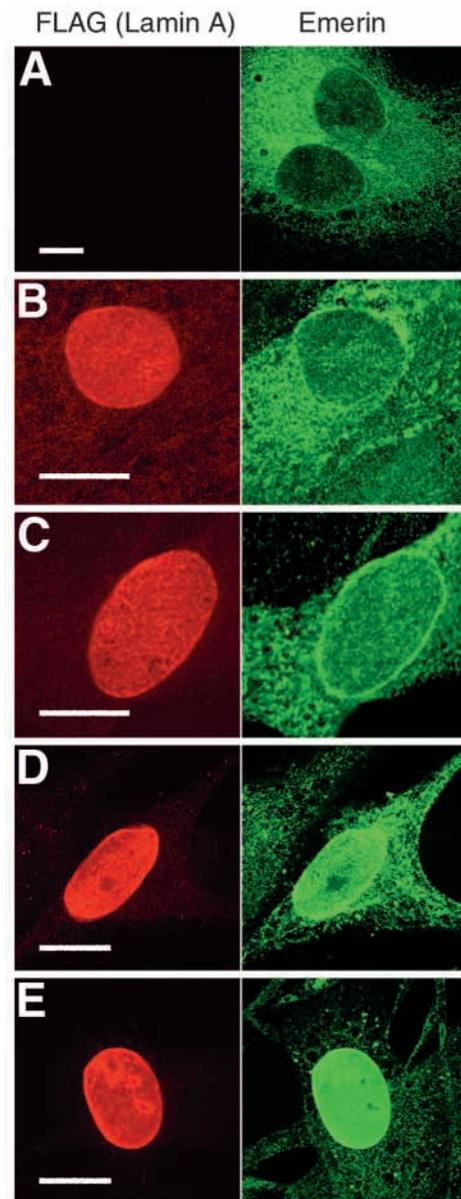


Fig. 2. Effects of lamin A transfection on the distribution of endogenous emerin in MEFs (*Imna*^{-/-}). (A) Untransfected cells show most of the emerin in the peripheral ER. A range of effects were observed for transfection with wild-type lamin A on the endogenous emerin: little or no effect on the distribution of emerin (B); partial relocation of emerin to the nucleus, with significant amounts of emerin remaining in the ER (C,D); and efficient relocation of emerin from the ER to the nucleus (E). All examples are wild-type lamin A in the pSVK3 plasmid. Bars, 10 μ m.

R386K). Because L85R specifically affects lamin C assembly (Raharjo et al., 2001), we could say that all five rod mutations cause defective assembly of the nuclear lamina.

Because most or all of these rod mutations cause lamin A to accumulate in intranuclear foci in about 10% of cells, we determined whether emerin colocalized in these foci. MEFs (*Imna*^{-/-}) were transfected with each mutant lamin A construct and examined by indirect immunofluorescence using antibody

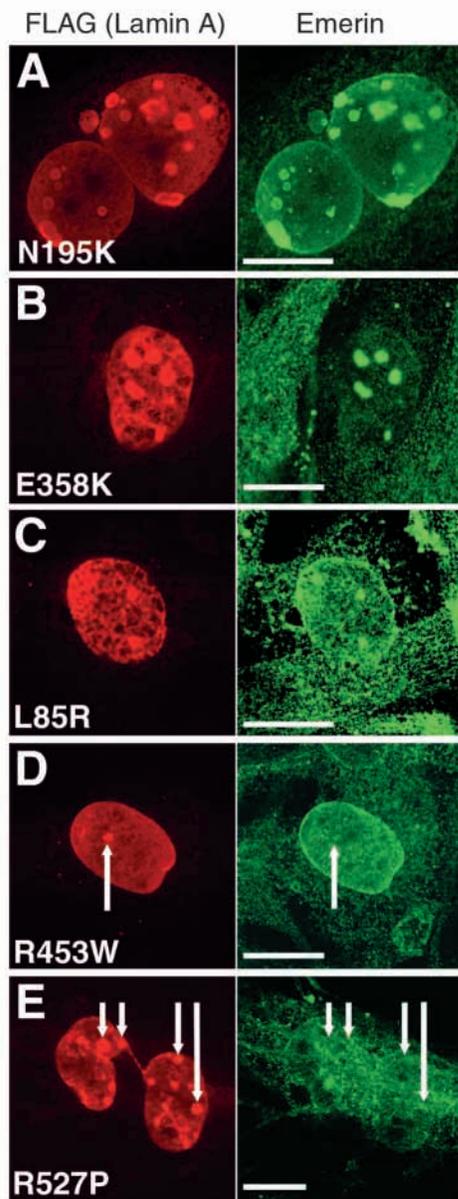


Fig. 3. Some mutant lamins form nuclear foci in MEFs (*Imna*^{-/-}) and emerin co-localizes with foci formed by ‘rod’ mutants but not ‘tail’ mutants. Transfection of MEFs (*Imna*^{-/-}) with lamin A N195K (A), E358K (B) or L85R (C) produced nuclear foci that were also positive for emerin (L85R produced foci only rarely; Table 1). Similar results were obtained with R386K and M371K (not shown). Two of the three ‘tail’ mutants, R453W (D) and R527P (E), produced nuclear foci only rarely and these did not accumulate emerin (arrows). Bars, 10 μ m.

against emerin (Fig. 3). The foci formed by four of the lamin A rod mutants were positive for emerin, N195K (Fig. 3A), E358K (Fig. 3B), M371K and R386K (data not shown). The fifth mutant, L85R, rarely produces lamin A foci, but these rare foci are also positive for emerin (Fig. 3C). Emerin staining was usually more intense in the larger foci (>1.5 μ m) but was also present in smaller foci.

Three mutations in the globular tail domain (R453W, W520S and R527P) produced abnormal foci rarely or not at all

(Table 1), but they did impair emerin relocalization (Table 1). The three-dimensional structure of this domain suggests that these mutations might disturb the overall structure of the domain and thus affect emerin binding (Dhe-Paganon et al., 2002; Krimm et al., 2002). The lipodystrophy R482Q mutation, by contrast, is on an outlying surface and might not disrupt the domain folding (Dhe-Paganon et al., 2002; Krimm et al., 2002), consistent with normal emerin relocalization after transfection (Table 1). Large nucleoplasmic foci were occasionally seen with R453W and R527P but emerin did not accumulate at these foci (Fig. 3D,E). This important difference from the rod mutants is consistent with the hypothesis that tail mutations, unlike rod mutations, act primarily by directly blocking emerin interaction.

Nuclear foci formed by lamin A rod mutants contain emerin

The fine detail of the emerin and lamin distribution in large foci was examined by collecting *z*-series of 0.25 μ m sections through the nuclei. Fig. 4A shows the red lamin A signal and the green emerin signal in one optical section of a MEF (*Imna*^{-/-}) transfected with N195K. The nuclear foci consist of transfected lamin A at the periphery with emerin trapped inside. Because the foci are very intensely stained by antibody, care was taken to avoid saturation of the digital image and loss of resolution by using the ‘SetCol LUT’ facility of the confocal microscope, as recommended by the manufacturers. In an *xz* section through one of the foci and the emerin is clearly inside a ring of transfected lamin A (Fig. 4B). Similar observations were made with the large nuclear foci formed by the other lamin A rod mutants. The large foci appear to occupy the entire thickness of the nucleus (1.5–3.5 μ m) and might be contiguous with the upper and lower nuclear surfaces, although it is difficult to establish this because the vertical resolution of the microscope is only 0.5–0.6 μ m, at best. Fig. 4C,D shows controls showing a MEF (*Imna*^{-/-}) transfected with N195K lamin A but with pre-immune rabbit serum in place of the anti-emerin serum, to confirm that the green fluorescence in Fig. 4A,B is authentic emerin. Two highly specific mAbs against emerin (Manilal et al., 1996) were also used to confirm the presence of emerin in nuclear foci of human HeLa cells transfected with the N195K mutant lamin A (Fig. 5). The mAb labelling experiment could not be performed on MEFs because the mAbs do not recognize mouse emerin. The emerin in the nuclear foci produced by lamin A rod mutants was not reported in earlier studies (Östlund et al., 2001; Raharjo et al., 2001), although the presence of endogenous A-type and B-type lamins was observed. Part of the explanation for this might be that, in smaller foci that do not traverse the whole nucleus (Fig. 4E–G), emerin tends to concentrate on the membrane side of the foci (asterisks in Fig. 4E, and compare the other two foci in Fig. 4E with Fig. 4F). Fig. 4E–G shows three confocal sections (top to bottom) of a nucleus in which one of the foci is associated with the lower part of the nucleus (arrow in Fig. 4G), whereas three other foci (lower right of Fig. 4E) are associated with the upper part of the nucleus. If this nucleus represents an early stage in the development of foci (and this is purely hypothetical, requiring time-lapse confirmation), we speculate that foci might grow from the upper or lower lamina and attract emerin, causing it to invaginate into the foci, possibly with its

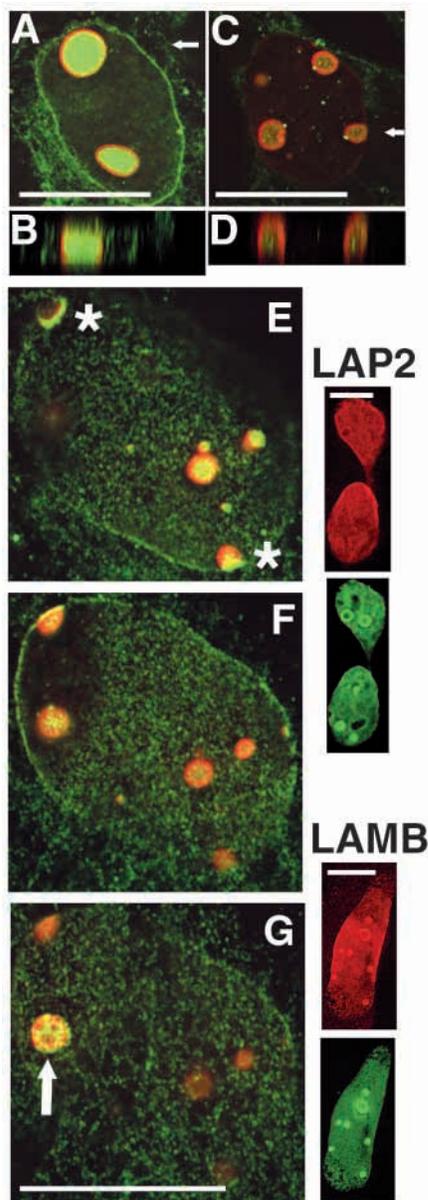


Fig. 4. Horizontal and vertical sections through nuclear foci in MEFs (*lmna*^{-/-}) transfected with N195K. A transfected nucleus with foci is shown from above (A) or in a side-on section (B) at the position shown by the arrows. The side-on view was created by taking 15 confocal sections 0.25 μm apart in a *z*-series and assembling them into a three-dimensional image with LaserVox software.

(A,B) Staining for transfected lamin A detected with anti-FLAG mAb (red) and endogenous emerin detected with anti-emerin serum (green). (C,D) Controls with identical laser settings, in which cells were stained with anti-FLAG mAb against lamin A (red) and pre-immune rabbit serum (green); in this case, the side-on view was built up from 11 *z*-series sections instead of 15. (E-G) Confocal sections (top to bottom) of a nucleus containing five foci. Two of these foci (asterisks in E) appear to be attached to the nuclear rim, where emerin is concentrated. Of the remaining three central foci visible in F, two appear to be attached to the upper surface (E) and one to the lower surface (arrow in G). The inset labelled LAP2 shows a double label with rabbit anti-FLAG for lamin A (green; ALEXA488 anti-rabbit Ig) and a commercial mAb against LAP2 (red; ALEXA546 anti-mouse Ig). The inset labelled LAMB shows a double label with monoclonal anti-FLAG for lamin A (green; FITC anti-mouse Ig) and a commercial goat antibody against lamin B (red; TRITC anti-goat Ig). Bars, 10 μm .

associated membrane. Fig. 4 also shows that the foci contain lamin B but very little LAP2, confirming previous observations (Östlund et al., 2001; Raharjo et al., 2001).

Transfected lamin A also colocalizes with abnormally distributed lamin B at the nuclear rim

Cells with a 'burst end' or 'cage' appearance at one pole of their nucleus occur frequently in *lmna*^{-/-} MEFs stained for B-type lamins (Sullivan et al., 1999). Transfected lamin A colocalizes with the lamin B in these abnormal nuclei and does not correct the unusual lamin B distribution (Fig. 6). The colocalization was independent of either the lamin A expression level or the mutation (data not shown). This result is consistent with assembly of an A-type lamina upon a pre-existing B-type lamina, in which case the mutations in lamin A do not appear to disrupt its assembly onto the B-type lamina.

Co-expression of wild-type lamin A reduces mislocalization of mutant lamin A into nuclear foci

In autosomal dominant laminopathies, mutant lamin A from one allele is invariably co-expressed with normal lamin A from the other allele in all cells of affected patients. To mimic this heterozygous condition in disease, cells [MEFs (*lmna*^{-/-}) or COS-7] were co-transfected with both wild-type (in pcDNA4 vector) and N195K mutant lamin A (in pSVK3 vector). The transfected protein products were detected by their different fusion tags (mouse anti-Xpress mAb and rabbit anti-FLAG polyclonal). High levels of co-transfection (>50%) were obtained by optimizing the electroporation conditions. In co-transfected cells, wild-type and mutant lamin A usually colocalized at the nuclear rim and in the nuclear interior (Fig. 7A). Of 400 co-transfected MEFs and COS cells examined, only two COS cells had large nuclear foci and these contained both wild-type and mutant lamins (Fig. 7B). Co-expression of the wild-type lamin A clearly reduced mislocalization of N195K lamin A into nuclear foci.

Discussion

Lamin A/C structure is made up of a small N-terminal region (amino acids 1-35) and a coiled-coil helical region (amino acids 35-390), followed by a linker region (amino acids 391-429) containing the nuclear localization signal (amino acids 417-422) and a C-terminal globular domain of known three-dimensional structure (amino acids 430-545) (Dhe-Paganon et al., 2002; Hutchison et al., 2001; Krimm et al., 2002). In lamin A, there is an additional C-terminal domain, absent from lamin C. The helical rod region is flanked by two serine-phosphorylation sites involved in lamin assembly/disassembly (reviewed in Hutchison et al., 2001).

In AD-EDMD/CMD1A, nearly all lamin A/C mis-sense mutations are found between amino acids 35 and 386 or between amino acids 442 and 541 (i.e. within the rod domain or the globular tail). No mis-sense mutations between amino acids 387 and 441 have been reported. The results of the

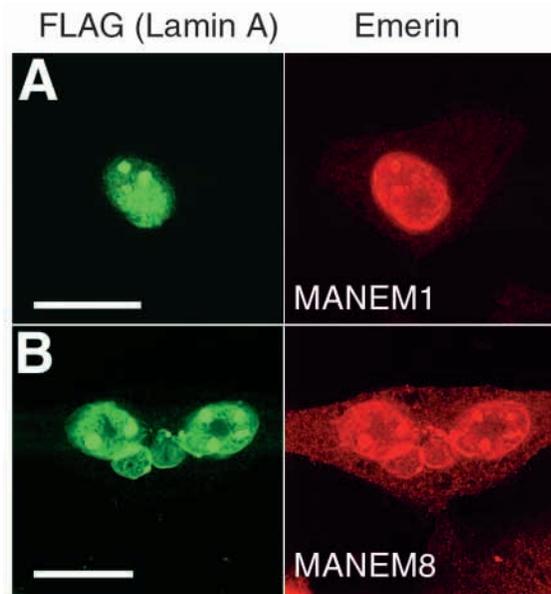


Fig. 5. Presence of emerin in nuclear foci in HeLa cells transfected with the N195K lamin A mutant. Nuclear foci of transfected lamin A, detected with anti-FLAG antibody, are shown to contain emerin using two different emerin-specific monoclonal antibodies, MANEM1 (A) and MANEM8 (B). Bars, 10 μ m.

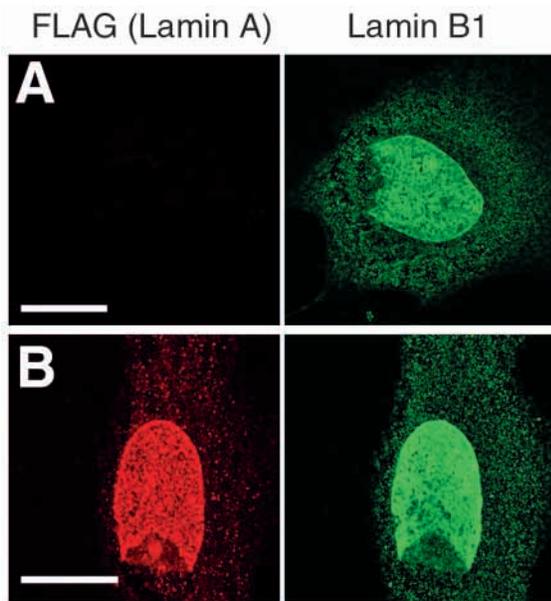


Fig. 6. Transfected lamin A colocalizes with endogenous lamin B1 at the nuclear rim in MEFs (*Imna*^{-/-}). Many nuclei in MEFs (*Imna*^{-/-}) showed 'burst ends', or abnormal lamin B1 distribution, at one pole (A). Transfected lamin A colocalized with lamin B1 in these cells (B). Double label with anti-FLAG mAb (red) and rabbit anti-lamin B1 (green). Bars, 10 μ m.

present study (Fig. 8) are consistent with the simple hypothesis that mutations in the rod domain cause defective lamin assembly, whereas tail-domain mutations impair the interaction of lamin A with emerin. Four out of five rod mutants of lamin A frequently produced abnormal nuclear foci

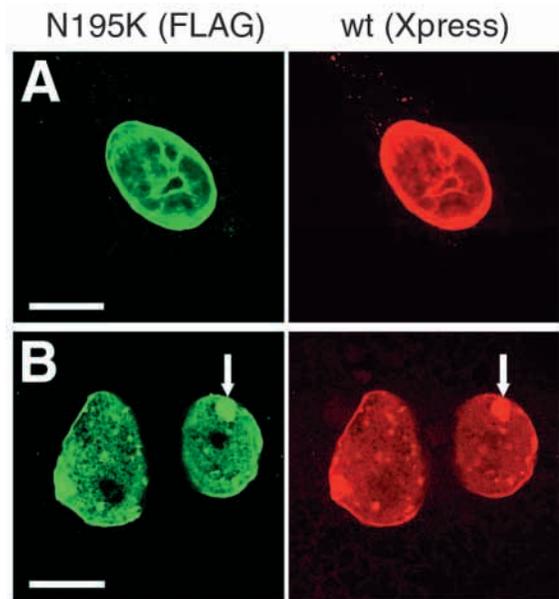
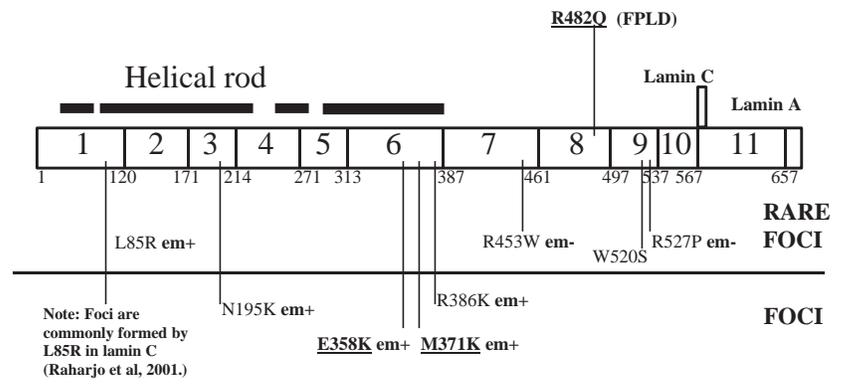


Fig. 7. Co-transfection with wild-type and mutant lamin A prevents or greatly reduces nuclear focus formation. MEFs (*Imna*^{-/-}) (A) and COS-7 cells (B) were co-transfected with pSVK3/lamin A N195K and pcDNA4/lamin A wild-type. The two lamins were detected with anti-FLAG polyclonal and anti-Xpress monoclonal antibodies, respectively. Nuclear foci were not seen with the MEFs (*Imna*^{-/-}). Nuclear foci (arrows) were very rarely seen with COS-7 cells (B) and these foci were positive for wild-type and mutant lamin A. Bars, 10 μ m.

in transfected cells (Fig. 8); the exception, L85R, rarely produced foci, but transfection with lamin C containing the L85R mutation has been shown to produce frequent foci (Raharjo et al., 2001). Frequent formation of abnormal foci is a likely symptom of defective lamina assembly. In all five cases, we found that the foci contained emerin, indicating that direct interaction with emerin was not impaired. Two of the three AD-EDMD tail mutants produced foci rarely, and these rare foci were negative for emerin, consistent with direct impairment of emerin interaction. The ability of lamin A to capture emerin at the nuclear rim when expressed in *Imna*^{-/-} MEFs clearly requires the ability to bind emerin directly and, indeed, all three tail mutants showed impaired ability to capture emerin at the nuclear rim. Two of the five rod mutants, E358K and M371K, were able to capture emerin as well as the control. Three rod mutants, however, showed reduced capture of emerin at the nuclear rim, although some capture of emerin still occurred. This suggests that there might be additional structural requirements for emerin capture by the lamina. Mutations causing structural changes that prevent a close approach of the lamin A molecule to the nuclear membrane, for example, might indirectly prevent emerin capture. A three-dimensional structure of the rod region of lamin A might throw light on the different emerin capture ability of rod mutations, in the same way that the three-dimensional structure of the lamin A tail domain has elucidated the special properties of the lipodystrophy mutations at R482 (Dhe-Paganon et al., 2002; Krimm et al., 2002). Some rod mutations cause changes in lamin C that are not evident in lamin A (Raharjo et al., 2001)

Fig. 8. Summary of results of transfection of MEFs with lamin A mutants. The 11-12 exons that encode lamins A and C are shown with amino acid numbers of approximate exon boundaries. The rod region is encoded by exons 1-6 and the common globular tail domain by exons 7-10. Shown below the diagram are mutations that cause EDMD/CMD1A, divided into those that commonly form nuclear foci ('FOCI') and those that rarely form nuclear foci ('RARE FOCI'). Those that form emerin-positive foci are 'em+', whereas those that form emerin-negative foci are 'em-' (foci were never seen for W520S). Above the diagram is the FPLD mutant, which neither formed foci nor affected emerin interaction. Mutants that retained the ability to recruit emerin to the nuclear rim in MEFs are shown in boldface and underlined.



so, if we view the lamina as a complex of lamins A, B and C, there might be defects in emerin capture by rod mutants *in vivo* that are not evident with transfected lamin A alone. It might be possible to use quantitative *in vitro* studies of emerin interaction with mutant lamins [e.g. BIAcore (Holt et al., 2001a) or pull-downs (Lee et al., 2001)] to distinguish direct effects of lamin A mutations on emerin binding from their indirect effects on emerin relocalization.

A crucial observation in the present study is that nuclear foci formed by lamin A rod mutants contain emerin, whereas foci formed by tail mutants do not. This differs somewhat from our earlier studies, which found no emerin in foci in mouse C2C12 myoblasts using the same rabbit anti-emerin serum (Östlund et al., 2001). Our monoclonal antibodies do not recognize mouse emerin. However, by transfecting HeLa cells, we could confirm the presence of endogenous human emerin in foci formed by the N195K mutant using specific mAbs against two different emerin epitopes (Fig. 5), although emerin staining was sometimes less intense than in MEFs (Fig. 5A). We are currently investigating differences between cell lines and culture/transfection conditions used in this and our earlier study that might affect emerin localization or alter the pathway of nuclear focus formation. Endogenous lamin A/C in normal cells might reduce the amount of emerin in nuclear foci compared with *lmna*^{-/-} MEFs. Time is another factor that might influence the number and composition of foci, all our studies having been done 48 hours after transfection.

The current view of lamina assembly is a sequential one, with B-type lamins followed by lamin A and, finally, lamin C (Raharjo et al., 2001; Vaughan et al., 2001). Our studies are consistent with this view. Thus, nuclei with B-type lamins absent from one pole, or abnormally distributed at one pole, occur in *lmna*^{-/-} MEFs (Sullivan et al., 1999) and transfected lamin A often followed a similar pattern. Lamin A enters nuclei through nuclear pores, whereas emerin in the ER enters around nuclear pores by diffusion and is usually captured at the nuclear rim by lamin A/C and/or other proteins (Östlund et al., 1999). Emerin and lamins, however, are also detected by antibodies in the nucleoplasm of cultured cells, often in discrete structures that might be invaginations of the nuclear membrane. The relationship between these small structures and the much larger mutant foci is not clear but it is possible that the latter form by a similar mechanism to the former or

that the former act as seeds for the growth of the latter. Invaginations of the nuclear membrane would be one way to explain how emerin with its hydrophobic transmembrane sequence can be found in the interior of the nuclear foci. In Fig. 4E-G, there is a clear indication that foci might begin to develop at the lamina, attracting emerin initially at the nuclear rim and eventually as invaginations into the interior of the foci. It is not clear whether such structures are really an early stage in the development of foci, but it might be possible to resolve this question by time-lapse studies of transfected GFP/lamin-A in living cells. More detailed analysis of the lipid and protein composition of nuclear foci and immunoelectron microscopy might also help us to understand how these structures develop and how they relate to normal lamina assembly.

A simplified conclusion about AD-EDMD/CMD1A phenotypes suggested by this study is that they might be caused by defective formation of the emerin/lamin-A/C complex. Some function of the complex that has yet to be identified but is of particular importance in skeletal and cardiac muscle, in which the diseases are manifested, is presumably affected (Morris, 2001). It must be remembered also that lamin A mutations reduce, rather than completely prevent, emerin relocalization and that abnormal nuclear foci are only formed in a small proportion of transfected cells. Furthermore, transfection of *lmna*^{-/-} MEFs mimics the homozygous situation, whereas autosomal dominant diseases are manifested in heterozygotes. Our co-transfection experiment of N195K and wild-type lamin A attempted to mimic the heterozygous state and abnormalities were rare. This is perfectly consistent with the fact that most tissues are unaffected in EDMD and CMD1A and that the characteristic cardiac abnormalities appear very late in human development (at least 10-20 years after birth, in most cases) (Morris, 2001). Mandibuloacral dysplasia is caused by a homozygous R527H mutation, quite similar to the R527P EDMD mutation studied here. The clinical features of this disease are quite unlike EDMD, affecting a wider range of tissues, although they often include FPLD features (Novelli et al., 2002). The good correlation between defective emerin/lamin-A complex formation and a range of EDMD/CMD1A mutations in lamin A/C strongly suggests that the explanation of the cardiac and skeletal muscle defects in EDMD/CMD1A lies in altered emerin-lamina interactions.

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