Properties of lamin A mutants found in Emery-Dreifuss muscular dystrophy, cardiomyopathy and Dunnigan-type partial lipodystrophy

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SUMMARY

Autosomal dominant Emery-Dreifuss muscular dystrophy is caused by mutations in the LMNA gene, which encodes lamin A and lamin C. Mutations in this gene also give rise to limb girdle muscular dystrophy type 1B, dilated cardiomyopathy with atrioventricular conduction defect and Dunnigan-type partial lipodystrophy. The properties of the mutant lamins that cause muscular dystrophy, lipodystrophy and dilated cardiomyopathy are not known. We transfected C2C12 myoblasts with cDNA encoding wild-type lamin A and 15 mutant forms found in patients affected by these diseases. Immunofluorescence microscopy showed that four mutants, N195K, E358K, M371K and R386K, could have a dramatically aberrant localization, with decreased nuclear rim staining and formation of intranuclear foci. The distributions of endogenous lamin A/C, lamin B1 and lamin B2 were also altered in cells expressing these four mutants and three of them caused a loss of emerin from the nuclear envelope. In the yeast two-hybrid assay, the 15 lamin A mutants studied interacted with themselves and with wild-type lamin A and lamin B1. Pulse-chase experiments showed no decrease in the stability of several representative lamin A mutants compared with wild-type. These results indicate that some lamin A mutants causing disease can be aberrantly localized, partially disrupt the endogenous lamina and alter emerin localization, whereas others localize normally in transfected cells.

Key words: Nuclear envelope, Lamins, Intermediate filaments, Muscular dystrophy, Lipodystrophy, Cardiomyopathy

INTRODUCTION

Emery-Dreifuss muscular dystrophy (EDMD) is characterized by 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). Emery-Dreifuss muscular dystrophy is inherited in both an autosomal dominant (AD-EDMD, OMIM 181350) and an X-linked, recessive (OMIM 310300) manner. In 1994, mutations in emerin, an integral protein of the nuclear envelope inner membrane, were shown to be responsible for X-linked EDMD (Bione et al., 1994; Manilal et al., 1996; Nagano et al., 1996). The connection between muscle disease and mutations in a nuclear envelope protein was unexpected, but was further established when Bonne et al. (Bonne et al., 1999) showed that AD-EDMD, a phenotypically indistinguishable syndrome, is caused by mutations in the LMNA gene, which encodes the nuclear envelope proteins lamins A and C. Mutations in LMNA have also been shown to cause limb girdle muscular dystrophy 1B (LGMD1B, OMIM 159001), a disease that differs from AD-EDMD by displaying a different distribution of affected skeletal muscle, a later occurrence of cardiac problems and an absence of early contractures (Muchir et al., 2000), and dilated cardiomyopathy without skeletal muscle pathology (OMIM 115200) (Fatkin et al., 1999; Bécane et al., 2000). LMNA mutations that cause cardiac and skeletal muscle disease are found throughout the gene, most being point mutations resulting in amino acid substitutions. There appears to be no clear correlation between the site of mutation and the severity of disease (Bonne et al., 2000). By contrast, mutations in LMNA clustering only in exons 8 and 11 have been shown to cause Dunnigan-type familial partial lipodystrophy (FPLD, OMIM 151660) (Cao and Hegele, 2000; Shackleton et al., 2000; Speckman et al., 2000; Vigouroux et al., 2000). FPLD is a rare, autosomal dominant disease characterized by the loss of subcutaneous adipose tissue from the extremities and trunk after the onset of puberty (Köbberling and Dunnigan, 1986).

Lamins are intermediate filament proteins that form the nuclear lamina, a meshwork on the nucleoplasmic side of the inner nuclear membrane (Aebi et al., 1986; Fisher et al., 1986; McKeon et al., 1986; Stuurman et al., 1998; Worman and Courvalin, 2000). Two general types of lamins have been identified in somatic cells, A-type and B-type. The somatic cell A-type lamins, lamin A, lamin C and lamin AΔ10, are alternative splice isoforms encoded by the LMNA gene (Lin and Worman, 1993; Machiels et al., 1996). Two different genes encode the somatic cell B-type lamins, lamin B1 and lamin B2 (Biamonti et al., 1992; Lin and Worman, 1995). Like all intermediate filament proteins, lamins have a conserved central rod-domain containing three α-helical segments, which is responsible for the formation of coiled-coil dimers. The rod-
domain is flanked by N-terminal head and C-terminal tail-domains, which vary significantly between different proteins (Steinert and Roop, 1988). The lamins bind to some of the integral membrane proteins of the inner nuclear envelope (Worman and Courvalin, 2000). Lamin A interacts with emerin and lamina associated polypeptide 1 (LAP1) in vitro (Foisner and Gerace, 1993; Clements et al., 2000; Sakaki et al., 2001).

Because the functions of lamins A and C are poorly understood, the mechanisms by which mutations cause different inherited diseases are not clear (Worman and Courvalin, 2000; Hutchison et al., 2001). Currently, it is not known if any of the disease-causing mutations of lamin A alter protein localization, lamina structure, protein-protein interactions or protein stability. We therefore investigated these properties of mutant forms of lamin A found in patients with inherited diseases.

MATERIALS AND METHODS

Plasmid construction

All cloning procedures were performed according to standard methods (Sambrook et al., 1989). For expression in mammalian cells, constructs that expressed FLAG-tagged polypeptides were made in pSVK3 (Amersham Pharmacia Biotech, Inc., Piscataway, NJ), which contains a multiple cloning site downstream from the SV-40 early promoter. cDNA encoding prelamin A, cloned into the BamHI and SalI restriction sites of plasmid pGBT9 (Ye and Worman, 1995), was excised by restriction endonuclease digestion with SalI and SalI and ligated into pBFT4, digested with the same restriction enzymes. pBFT4 is a pBluescript II KS- (Stratagene, La Jolla, CA) based plasmid containing a Kozak sequence, ATG and the FLAG-tag coding sequence downstream from the SV-40 early promoter. The resulting plasmid was digested with SpeI and XhoI to excise a cDNA encoding ATG-FLAG-prelamin A that was ligated into pSVK3, digested with XhoI (an isochozyme of SpeI) and XhoI.

cDNAs encoding mutant forms of prelamin A, except the ΔNLA mutant, which lacked amino acids 1-33, were made using the Transformer™ Site-Directed Mutagenesis Kit (CLONTech Laboratories, Inc., Palo Alto, CA), following the manufacturer’s instructions. ΔNLA was generated by polymerase chain reaction (Saiki et al., 1987), using the Gene Amp PCR System 2400 (Applied Biosystems, Foster City, CA), with a Smal restriction site engineered at the 5’ end of the sense primer and an antisense primer spanning the SalI site found at nucleotide 746 of prelamin A cDNA. Reaction products were digested with Smal and SalI and ligated into prelamin A-pBGT9 digested with the same enzymes. The resulting plasmid was digested with SalI and SalI and ligated into the Smal and SalI restriction sites of pSVF, a vector constructed by insertion of the FLAG-epitope between the EcoRI and KpnI restriction sites of pSVK3.

For studies with the yeast two-hybrid system, wild-type prelamin A and lamin B1 cDNAs in vectors pGAD424 and pGBT9 and lamin B1 cDNA in vector pGBT9 were those previously described (Ye and Worman, 1995). cDNAs obtained by site-directed mutagenesis were excised from vector pSVK3 using the restriction endonucleases Smal and SalI and ligated into pGBT9 and pGAD424 digested with the same enzymes. ΔNLA was inserted into pGBT9 as described above and then digested with Smal and SalI and ligated into the Smal and SalI restriction sites of pGAD424. All cDNAs were sequenced using an ABI Prism 377 automated sequencer (Applied Biosystems).

Cell culture, transfection and immunofluorescence microscopy

C2C12 cells (a gift from Hal Skopicki, Columbia University, New York, NY) were grown in Dulbecco’s modified Eagle medium (D-MEM) containing 10% fetal bovine serum (Life Technologies, Gaithersburg, MD) at 37°C and 10% CO2. Cells were transfected in chamber slides using Lipofectamine PLUS™ (Life Technologies), following the manufacturer’s instructions. The cells were overlaid with the lipid-DNA complexes for approximately 23 hours, the first five of which were in serum-free medium. The cells were then allowed to grow in fresh medium for 24 hours post-transfection before preparation for immunofluorescence microscopy.

Fixation and labeling of cells for immunofluorescence microscopy were performed as described previously (Östlund et al., 1999). The monoclonal primary antibodies used were anti-FLAG M5 (Sigma, St Louis, MO) diluted 1:200, anti-pCA (proliferating cell nuclear antigen) P10 (Roche Molecular Biochemicals, Indianapolis, IN) diluted 1:20, anti-nuclear pore complex MAb414 (a gift from Tarik Soliman, Laboratory of Cell Biology, Rockefeller University, New York, NY) diluted 1:5,000 and anti-lamin B2 X223 (a gift from Georg Krohne, University of Würzburg, Würzburg, Germany) diluted 1:400. Polyclonal primary antibodies were used as anti-lamin B1 (Cance et al., 1992) at a dilution of 1:2,000, anti-lamin A/C (Cance et al., 1992) at a dilution of 1:1,000, anti-emerin (a gift from Glenn Morris, North East Wales Institute, Wrexham, UK) at a dilution of 1:3,000 and anti-LAP2 (a gift from Katherine Wilson, Johns Hopkins University, Baltimore, MD) at a dilution of 1:500. Secondary antibodies used were Rhodamine Red™, X-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), Alexa™ 568-conjugated goat anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.). For DNA-staining, 20 μg/ml propidium iodide (Sigma) was added to the secondary antibody incubation. Labeled and washed slides were dipped in methanol, air-dried, and coverslips were mounted using ProLong Gold Antifade Kit (Molecular Probes, Inc.) or SlowFade Light Antifade Kit (Molecular Probes, Inc.). 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).

Yeast two-hybrid assay

Saccharomyces cerevisiae strain Y187 (Clontech Laboratories, Inc.) was transformed with plasmids encoding lamins fused to the DNA binding domain of the S. cerevisiae GAL4 protein (pGAD9) or to the GAL4 transcriptional activation domain (pGAD424). Transformations and β-galactosidase assays were done according to the Matchmaker Two-Hybrid System manual (Clontech Laboratories, Inc.).

Pulse-chase assay

COS-7 cells were grown in D-MEM containing 10% fetal bovine serum (Life Technologies) at 37°C and 5% CO2. The cells were transfected using Lipofectamine PLUS™ (Life Technologies), following the manufacturer’s instructions. The cells were overlaid with the lipid-DNA complexes for approximately 23 hours, the first five of which were in serum-free medium, and were then grown in fresh medium for 24 hours before being subcultured at a 1:5 dilution into 60 mm tissue culture dishes. After another 24 hours, cells were washed with D-MEM without methionine and then overlaid with 1 ml D-MEM without methionine, containing 28 μCi Pro-mix [-35S] in vitro cell labeling mix (Amersham Pharmacia Biotech, Inc.). After 1 hour, cells were washed twice with phosphate-buffered saline (PBS) and either incubated in non-radioactive D-MEM with 10% fetal bovine serum for an additional 3 or 25 hours or lysed immediately. For cell lysis, cells were washed three times in PBS and incubated for 40 minutes at 4°C in 440 μl lysis buffer (50 mM Tris-HCl, pH 8, 5 mM ethylenediaminetetraacetic acid, 0.1% sodium dodecyl sulfate

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(SDS), 1% Triton X-100) with 2% bovine serum albumin (BSA), 0.5 mM phenylmethylsulfonyl fluoride and 4.4 μl protease cocktail inhibitor (Sigma). The cells were then scraped off with a rubber policeman and sheared six times through a 21-gauge needle. After centrifugation for 10 minutes at 10,000 g, the supernatant was incubated overnight at 4°C with 30 μl anti-FLAG M2-agarose affinity gel (Sigma). The affinity gel was collected by centrifugation for 2 minutes at 325 g, washed three times in lysis buffer with 2% BSA, once with lysis buffer without BSA, once with 100 mM Tris-HCl (pH 6.8) and 0.5 M NaCl, and once with 100 mM Tris-HCl (pH 6.8). For the first, fourth and fifth washes, the gel was incubated for 5 minutes on a rotating wheel at 4°C. Twenty μl SDS-sample buffer (Laemmli, 1970) was added to the affinity gel and the samples were incubated for 15 minutes at 70°C and centrifuged for 2 minutes at 325 g. The proteins in the supernatants were separated by SDS-polyacrylamide gel electrophoresis (PAGE). Gels were fixed for 30 minutes in 35% methanol, 10% acetic acid, incubated 30 minutes with Amplify (Amersham Pharmacia Biotech, Inc.), dried and exposed to Hyperfilm ECL (Amersham Pharmacia Biotech, Inc.) at −70°C.

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

Some lamin A mutants have a dramatically abnormal intranuclear localization

To determine if the intracellular localizations of mutant forms of lamin A differ from that of the wild-type, we transiently transfected C2C12 mouse myoblasts with plasmids expressing wild-type and mutated forms of prelamin A. Prelamin A is a precursor form of mature lamin A, which is processed by endoproteolytic cleavage of the final 18 amino acids (Weber et al., 1989; Sinensky et al., 1994). For detection of expressed proteins, FLAG-epitopes were fused to the N-termini of the constructs. The mutations studied were: R60G, L85R, N195K and E203G, found in patients with dilated cardiomyopathy (Fatkin et al., 1999); E358K, M371K, R386K, R453W, W520S, R527P, T528K and L530P, identified in patients with AD-EDMD (Bonne et al., 1999; Bonne et al., 2000); and R482Q, R482W and K486N, found in patients with FPLD (Cao and Hegele, 2000; Shackleton et al., 2000) (Fig. 1A).

In transfected cells, FLAG-tagged wild-type lamin A localized to the nuclear periphery, showing colocalization with lamin B1, a marker for the lamina and nuclear envelope (Fig. 1B). A majority of the FLAG-tagged lamin A mutants showed no gross abnormalities in cellular localization; however, four of the mutants, N195K, E358K, M371K and R386K, showed a dramatically aberrant localization in many cells, accumulating in large intranuclear foci (Fig. 1B). These four mutants often showed a decrease in nuclear rim localization, giving a more diffuse, nucleoplasmic staining, which was also seen in cells lacking intranuclear foci.
(Fig. 1B, insets). The diffuse, nucleoplasmic staining was particularly common for mutant R386K, with approximately 95% of transfected cells with or without large intranuclear foci showing this pattern. Mutants N195K, E358K and M371K showed a mostly diffuse, nucleoplasmic localization in 50-70% of transfected cells.

Not all cells expressing the four foci-forming lamin A mutants contained large intranuclear foci. We counted 200 transfected cells of each type were studied in each experiment. Nuclear foci smaller than 0.7 μm were not counted, as lamin foci smaller than this size frequently can be seen in all cells, including untransfected cells. Asterisks (*) denote statistically significant differences between mutant and wild-type (WT) cells in the number of cells with nuclear foci as determined by χ²-test (P<0.001). In all other cases, there were no significant differences (P>0.05).

### Table 1. Statistical analysis of nuclear foci in cells expressing lamin A mutants

<table>
<thead>
<tr>
<th>Cells without foci</th>
<th>Cells with foci</th>
<th>Cells with foci (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>199.33±0.33</td>
<td>0.67±0.33</td>
</tr>
<tr>
<td>R60G</td>
<td>199.33±0.66</td>
<td>0.67±0.66</td>
</tr>
<tr>
<td>L85R</td>
<td>199.00±0.58</td>
<td>1.00±0.58</td>
</tr>
<tr>
<td>N195K*</td>
<td>164.33±6.44</td>
<td>35.67±6.44</td>
</tr>
<tr>
<td>E203G</td>
<td>198.67±0.33</td>
<td>1.33±0.33</td>
</tr>
<tr>
<td>E358K*</td>
<td>177.00±3.05</td>
<td>23.00±3.05</td>
</tr>
<tr>
<td>M371K*</td>
<td>135.33±8.29</td>
<td>64.67±8.29</td>
</tr>
<tr>
<td>R386K*</td>
<td>155.00±5.83</td>
<td>45.00±5.83</td>
</tr>
<tr>
<td>R453W</td>
<td>196.67±1.45</td>
<td>3.33±1.45</td>
</tr>
<tr>
<td>R482Q</td>
<td>199.00±0.58</td>
<td>1.00±0.58</td>
</tr>
<tr>
<td>R482W</td>
<td>199.00±0.58</td>
<td>1.00±0.58</td>
</tr>
<tr>
<td>K486N</td>
<td>198.33±0.88</td>
<td>1.67±0.88</td>
</tr>
<tr>
<td>W520S</td>
<td>197.67±0.88</td>
<td>2.33±0.88</td>
</tr>
<tr>
<td>R527P</td>
<td>195.33±1.45</td>
<td>4.67±1.45</td>
</tr>
<tr>
<td>T528K</td>
<td>196.67±0.66</td>
<td>3.33±0.66</td>
</tr>
<tr>
<td>L530P</td>
<td>198.33±0.88</td>
<td>1.67±0.88</td>
</tr>
<tr>
<td>ΔNLA*</td>
<td>113.00±2.64</td>
<td>87.00±2.64</td>
</tr>
</tbody>
</table>

Numbers shown are means from three experiments±s.e. unless otherwise stated. 200 transfected cells of each type were studied in each experiment.

Immunofluorescence analysis of cells expressing foci-forming lamin A mutants

To investigate further the composition of the abnormal nuclear foci formed by four of the lamin A mutants and their downstream effects on the nuclear envelope, we used several different markers to perform a detailed immunofluorescence microscopy analysis. Mislocalization of the lamin A mutants was often accompanied by disturbances in the localization of endogenous lamin B1. In many cells, lamin B1 was found in the interior of the intranuclear foci, with the mutant lamin A being more concentrated at the edges (Fig. 2). This phenotype was seen to a variable degree, being more frequent with mutants E358K and R386K (approximately 90% of cells with foci), where the foci often were large, than with mutants N195K (20-50%) and M371K (10-50%). The mislocalization of lamin B1 was, however, not complete, and in virtually all cells, some lamin B1 could still be detected at the nuclear periphery (Fig. 2, arrows). In occasional cells, lamin B1 was missing from one pole of the nucleus (Fig. 2, mutant...
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N195K), or from buds protruding out from the nucleus. This phenotype has been described in cells from mice lacking A-type lamins (Sullivan et al., 1999) and fibroblasts from patients with FPLD (Vigouroux et al., 2001). A similar pattern of nuclear foci was observed previously when a mutant form of lamin A (ANLA), lacking the nonhelical head-domain (amino acids 1-33), was microinjected into mammalian cells or added to Xenopus laevis interphase egg extracts (Spann et al., 1997). When FLAG-tagged ANLA was expressed in our system, the same pattern was seen in many cells (Fig. 2) (Table 1).

Lamin B2 was also partly mislocalized in some cells with mutant lamin A-containing nuclear foci. Representative results for the lamin A E358K mutant and ANLA are shown in Fig. 3A. Although apparently slightly less affected than lamin B1, lamin B2 was sometimes missing from one pole of the nucleus or concentrated near the nuclear foci (Fig. 3A, arrows). However, the peripheral distribution of the integral inner nuclear membrane protein LAP2b appeared normal in all cells (Fig. 3B).

We also examined the distribution of nuclear pore complexes in cells containing aberrant lamin foci. Nuclear pore complexes showed a normal localization, even in cells with large intranuclear lamin foci (Fig. 4). They were not present in the foci, even when the foci were situated near the nuclear periphery (Fig. 4, N195K). As for lamin B1, there were occasional cells where the nuclear pore complexes were excluded from one pole of the cell (Fig. 4, M371K), as has also been reported in mouse cells lacking A-type lamins and fibroblasts from patients with FPLD (Sullivan et al., 1999; Vigouroux et al., 2001).

To obtain additional information about the effects of foci-forming lamin A mutants on the nuclear lamina, we performed double labeling with anti-FLAG antibodies, recognizing the exogenous lamin A, and anti-lamin A/C antibodies, recognizing the exogenous protein as well as endogenous lamins A and C. This analysis showed an accumulation of endogenous lamins A and C inside the foci, similar to the localization frequently seen with endogenous lamin B1 (Fig. 5). As with lamin B1, however, some lamin A/C still remained at the nuclear periphery in the majority of the cells. In two separate experiments with 100 transfected cells counted, peripheral lamin A/C was present in 85-100% of cells expressing mutants N195K, E358K and M371K, and 70-90% of cells expressing mutants R386K and ANLA (Fig. 5, arrows).

Lamin A has been shown to bind to emerin in vitro (Clements et al., 2000; Sakaki et al., 2001) and, in cells from mice lacking A-type lamins, emerin is partly mislocalized to the cytoplasm (Sullivan et al., 1999). To investigate whether mutant forms of lamin A caused a mislocalization of emerin in the presence of wild-type lamin A, the situation in heterozygous patients, we stained C2C12 cells expressing the foci-forming lamin A mutants with antibodies against emerin (Fig. 6A). A loss of emerin from the nuclear envelope was seen in a subset of all transfected cells, also some cells transfected with wild-type lamin A. Similar results have been reported in transfected HeLa cells by Raharjo et al. (Raharjo et al., 2001). All foci-forming mutants except E358K led to a loss of emerin from the nuclear envelope in a significantly greater number of cells than wild-type lamin A (Fig. 6B). We also investigated loss of emerin from the nuclear envelope in cells expressing three mutants that do not form foci (R60G, E203G and K486N). None of these mutants led to an increased loss of emerin from the nuclear envelope, compared to wild-type (data not shown). Emerin, similar to nuclear pore complex proteins and LAP2, did not localize to the intranuclear lamin foci, further indicating that the foci are not
membrane invaginations of the nuclear envelope but separate structures inside the nucleus.

Finally, we examined the association of PCNA and DNA with the aberrant lamin foci. PCNA, a cofactor of DNA polymerase δ, which was shown previously to localize inside intranuclear foci containing ΔNLA in Xenopus egg extract experiments (Spann et al., 1997), very rarely accumulated inside the lamin A foci in our experiments (data not shown). DNA appeared to be mostly excluded from the foci (data not shown).

**Lamin A mutants interact with other lamins in the yeast two-hybrid assay**

Lamins, like other intermediate filaments, form parallel dimers through coiled-coil interactions between their α-helical rod-domains (Stuurman et al., 1998). To investigate if the ability of mutant lamin A molecules to interact was impaired, we performed a two-hybrid assay. Lamin A, prelamin A, lamin B1 and lamin C have previously been shown to interact with themselves and with each other in the yeast two-hybrid system (Ye and Worman, 1995). In this system, an interaction between a protein fused to the GAL4 activation domain and a protein fused to the GAL4 DNA-binding domain causes the expression of β-galactosidase, which can be measured using a colorimetric assay (Fields and Song, 1989). We expressed the 15 mutant forms of prelamin A described above as fusion proteins with the GAL4 activation domain, or the GAL4 DNA-binding domain, in S. cerevisiae. All of the mutant proteins interacted with themselves, wild-type prelamin A and wild-type lamin B1 (data not shown). No differences were seen between the mutant and wild-type lamins in the two-hybrid interaction assay. Although it is not possible using this assay to determine whether the protein-protein interactions formed are the coil-coiled ones in the normal lamina, these results suggest that the primary lamin-lamin dimerization is not disturbed in these mutants.

**Fig. 4.** Nuclear pore complexes are not present in nuclear foci. The panels show laser scanning confocal immunofluorescence microscopy images of C2C12 cells transfected with prelamin A with missense mutations as indicated. Short arrows indicate lack of pore complexes in the nuclear foci and long arrows their absence from one pole of some nuclei. Antibodies used were monoclonal antibodies against nuclear pore complex protein p62 (NPC) (left panel) and polyclonal antibodies against lamin A/C (middle panel). The monoclonal antibodies were recognized by FITC-conjugated secondary antibodies and the polyclonal antibodies were recognized by rhodamine-conjugated secondary antibodies. The panels to the right show an overlay of the FITC (green) and rhodamine (red) channels. Bars, 10 μm.

**Fig. 5.** Endogenous lamin A/C is localized both to the nuclear foci and to the nuclear rim. Staining with monoclonal antibodies against FLAG, recognizing exogenous protein, and polyclonal anti-lamin A/C antibodies, recognizing both exogenous and endogenous A-type lamins. The monoclonal antibodies were recognized by FITC-conjugated secondary antibodies and the polyclonal antibodies were recognized by rhodamine-conjugated secondary antibodies. The panels show an overlay of the FITC (green) and rhodamine (red) channels. Areas of colocalization appear yellow. Arrows show lamin A/C localized to the nuclear envelope. Bars, 10 μm.
Several lamin A mutants are as stable as wild-type lamin A
To investigate whether representative mutant lamin A proteins were less stable than wild-type lamin A, we performed pulse-chase analyses. COS-7 cells were transfected with cDNAs encoding wild-type or mutant prelamin A with FLAG-epitopes fused to their N-termini. The cells were pulsed with $[^{35}S]$-methionine for 1 hour and then harvested or grown in nonradioactive media for 3 or 25 hours. Cell lysates were incubated with anti-FLAG M2-agarose affinity gel and the immunoprecipitates were separated by SDS-PAGE and exposed to Hyperfilm ECL. Representative results are shown in Fig. 7. At time-point zero (no chase), both prelamin A and the slightly smaller mature lamin A could be seen in all samples from transfected cells. After 3 hours of growth in nonradioactive media, no prelamin A could be seen and the total levels of radiolabeled FLAG-tagged lamin A had decreased; it could, however, still be detected in all cases. After 25 hours of incubation, very small amounts of radiolabeled lamin A could still be detected in all samples. These results showed that the representative lamin A mutants N195K, M371K, R386K, R453W, R482W and K486N were as stable
Fig. 7. Pulse-chase analysis of wild-type and mutant forms of lamin A. COS-7 cells were transiently transfected with wild-type (WT) or mutant forms of prelamin A containing FLAG-epitopes, as indicated above the panels. The cells were pulse-labeled with [35S]-methionine for 1 hour. After 0, 3 or 25 hours (indicated above the panels) of incubation in non-radioactive media (chase), cell lysates were prepared and immunoprecipitated using M2 anti-FLAG agarose. The panels show autoradiograms of precipitated proteins separated by SDS-PAGE. Migration of molecular mass markers is indicated on the left. Plus signs (+) show prelamin A; asterisks (*) show mature lamin A. All experiments were performed in triplicate; representative samples are shown. Owing to the large number of samples and the importance of rapid handling, not all samples could be prepared at once. The results from different experiments varied slightly in labeling efficiency and level of background. The first five panels and the last three panels are taken from two separate experiments. Immunoprecipitates from transfected cells also contained a protein with an apparent molecular mass of 100 kDa. The identity of this protein is not clear but it was absent in untransfected cells and recognized by anti-FLAG and anti-lamin A/C antibodies on western blots (data not shown), suggesting that it is an aberrantly expressed form of lamin A.

as wild-type lamin A. Mutant R386K seemed slightly more stable than the others after 3 hours of chase. Wild-type lamin A and these lamin A mutants were also processed normally from prelamin A to the mature protein.

DISCUSSION

We have investigated 15 lamin A mutants in patients with dilated cardiomyopathy, AD-EDMD and FPLD. These mutations are dominant and patients carry one mutant and one wild-type copy of the LMNA gene. There are many possible mechanisms for how these mutations may cause disease, such as changes in the stability, localization and interactions of the mutant proteins.

Mutant lamins are as stable as wild-type lamins

Disease phenotypes may be caused by haploinsufficiency of lamins A and C due to low levels of expression or rapid degradation of the mutant lamin protein. One mutation causing AD-EDMD is a nonsense mutation at the codon for amino acid six (Bonne et al., 1999; Bécane et al., 2000); in this case a reduction of lamins A and C most likely caused the disease. In patients with this mutation, both lamin A/C and emerin were correctly localized to the nuclear envelope as shown by immunolabeling of a cardiac biopsy (Bonne et al., 1999), although there was a decrease in the amount of lamin A/C in cardiac tissue, as shown by western blot (Bécane et al., 2000). Our studies indicate no decrease in the stability of several mutant lamins, including those in AD-EDMD, cardiomyopathy and FPLD. Therefore, a simple haploinsufficiency of lamins A can not be the only pathophysiological mechanism. Studies of mutant lamins A and C in fibroblasts from patients carrying the LMNA mutations causing R482Q and R482W substitutions showed these proteins to be present at similar levels to the wild-type proteins in control cells (Vigouroux et al., 2001). Further studies of the levels of mutant lamins A and C in cells from patients carrying other LMNA mutations would provide important confirmation of our results on the stability of the mutant proteins.

Mutations in the lamin A rod-domain can cause an abnormal intranuclear localization of the protein

Mutant proteins may be unable to carry out their normal functions, or disrupt the functions of associated proteins, because of mislocalization in cells. We examined the intracellular localization of 15 mutant lamin A proteins by immunofluorescence microscopy. The most striking feature in these studies was the formation of intranuclear foci by four of the lamin A mutants (N195K, E358K, M371K and R386K), which was often accompanied by a mislocalization of some of the endogenous lamins. These cells also had a decrease in the localization of the mutant lamins to the nuclear periphery. A nucleoplasmic localization and formation of intranuclear foci have also been reported when the N195K lamin A mutant was expressed in HeLa cells (Raharjo et al., 2001). The four lamin proteins that formed intranuclear foci all had mutations introducing a lysine into the rod-domain. Three out of the four proteins had mutations at the end of the central, α-helical rod-domain, which is evolutionary well conserved between all intermediate filaments (Stuurman et al., 1998). A lamin A mutation (R377H) causing LGMD1B has also recently been mapped to this region (Muchir et al., 2000). The conserved segments of the rod-domain have been shown to play crucial roles in the assembly of intermediate filament dimers into higher order oligomers (Stuurman et al., 1998). Mutations in the corresponding regions of keratin K5 and K14 are responsible for the majority of skin diseases caused by keratin mutations, for example the Dowling-Meara type of epidermolysis bullosa simplex (McLean and Lane, 1995). This disease and others caused by keratin mutations are blistering skin
disorders resulting from epithelial fragility. It is conceivable that mutations in lamins A and C cause nuclear fragility. Nuclei of embryonic fibroblasts from mice lacking A-type lamins and *Xenopus* nuclei assembled in egg extracts with the ΔNLA mutant have been shown to be more fragile than wild-type nuclei (Spann et al., 1997; Sullivan et al., 1999). One hypothesis is that an increased fragility of nuclei can be particularly harmful to muscle cells, where they are subjected to mechanical stress (Worman and Courvalin, 2000; Hutchison et al., 2001). This could explain the muscle-specific nature of AD-EDMD and cardiomyopathy but does not explain why the specialized cardiomycocytes involved in atrioventricular conduction may be more readily affected than contractile cardiomycocytes (Morris, 2000).

The ‘nuclear stress-hypothesis’ as a pathophysiological mechanism of disease is less likely in the case of FPLD. The restriction of missense mutations causing FPLD to two regions in exon 8 and 11 implies that they encode a domain important for a specific lamin A function, for example interaction with a protein important for adipocyte survival. This is further suggested by the concentration of the majority of FPLD mutations to codon 482 (Cao and Hegele, 2000; Shackleton et al., 2000; Speckman et al., 2000; Vigouroux et al., 2000). In our studies, as well as in the studies by others (Holt et al., 2001; Raharjo et al., 2001), all using transiently transfected cells, there were no gross abnormalities in the localization or stability of lamin A-containing mutations found in FPLD. However, fibroblasts from FPLD patients with the R482Q and R482W lamin A mutations, and some transfected fibroblasts overexpressing the R482W mutant, show more subtle nuclear abnormalities (Vigouroux et al., 2001).

**Effects of lamin mutations on protein-protein interactions**

To examine the ability of mutant lamins to self-interact and to interact with wild-type lamins, we used the yeast two-hybrid assay. Our results showed no indication of impaired lamin-lamin interactions at this level. However, the two-hybrid assay does not show whether the complexes formed are the usual coiled-coil dimers. Neither would it detect disturbances of higher order lamin interactions, such as filament formation. Subtle defects of lamin filament formation are at this time difficult to detect because lamins, as opposed to cytoplasmic intermediate filaments, do not form stable filaments in vitro.

Lamin A binds to LAP1 and emerin (Foisner and Gerace, 1993; Clements et al., 2000; Sakaki et al., 2001). The interaction between lamin A and emerin is especially intriguing because mutations in these two proteins both cause the EDMD phenotype. In cells from mice lacking A-type lamins, emerin is partially mislocalized to the cytoplasm (Sullivan et al., 1999). These mice show typical signs of EDMD. Heterozygous mice, having only one wild-type copy of the *LMNA* gene, have a slight mislocalization of emerin but are healthy (Sullivan et al., 1999). We have studied the localization of emerin in C2C12 cells expressing mutant forms of lamin A. Our studies showed an increased loss of endogenous emerin from the nuclear envelope when C2C12 cells, which have wild-type lamin A in addition to the mutant forms, were transfected with mutants N195K, M371K, R386K or ΔNLA. However, as cells transfected with wild-type lamin A also exhibited a loss of emerin from the nuclear envelope, although to a significantly lower extent, it is not clear whether the emerin loss was due to specific disturbances in the interactions between emerin and mutant lamins or to a loss of peripheral lamins caused by the mutants. Alternatively, other effects of lamin A overexpression in cells could lead to emerin mislocalization, but whatever these effects may be, certain lamin A mutants enhance them. An increased loss of emerin from the nuclear envelope has also been reported in HeLa cells expressing the lamin A mutants L85R, N195K and L530P, compared with wild-type (Raharjo et al., 2001).

**Do lamins have a role in gene expression and DNA replication?**

Alternative hypotheses, which could explain the tissue-specific nature of the diseases caused by mutations in the A-type lamins, are that these proteins are involved in specific gene expression or DNA replication events, or both. A-type lamins have been shown to bind proteins involved in transcriptional regulation such as the retinoblastoma protein (Mancini et al., 1994), and ectopic expression of lamin A has also been shown to induce muscle-specific genes in undifferentiated cells (Lourim and Lin, 1992). A role for the inner nuclear membrane in the regulation of gene expression has previously been suggested by the interaction between the lamin B receptor, an integral protein of the inner nuclear membrane, and human orthologues of *Drosophila* HP1 (Ye and Worman, 1996; Ye et al., 1997). In *Drosophila*, HP1 suppresses the expression of normally active euchromatic genes translocated near heterochromatin (Eissenberg et al., 1990). Several other nuclear proteins involved in neuromuscular disease are involved in gene expression, either at the level of transcription, splicing or mRNA transport (Morris, 2000). The nuclear lamins are also required for DNA replication during the S-phase and the lamin foci formed in *Xenopus* egg extracts containing the ΔNLA mutant were previously shown to contain the DNA polymerase δ cofactor PCNA (Spann et al., 1997). PCNA, however, did not accumulate in the foci formed by lamin mutants in C2C12 cells in our studies. The reason for this discrepancy is not clear; one possibility is that PCNA localization is dependent on cell-cycle phase. Although in vitro assembled *Xenopus* nuclei are in S-phase, it is not clear whether cells containing nuclear foci can enter S-phase. It may also be due to different fixation techniques; our cells were fixed with methanol, which yields a staining pattern where only PCNA bound to specific nuclear structures is recognized by the antibody (Bravo and Macdonald-Bravo, 1987). There may also be differences in lamina structure between mammalian cells versus *Xenopus* egg extracts, which has been suggested by Izumi et al. (Izumi et al., 2000); they showed that PCNA is not colocalized with ΔNLA foci in CHO cells.

The recent results of others (Raharjo et al., 2001; Vigouroux et al., 2001) and those reported in this study provide a starting point towards understanding the properties of disease-causing lamin A/C mutants. They also exclude some possible pathogenetic mechanisms, such as haploinsufficiency of lamin A, absence of prelamin A processing or cytoplasmic sequestration, as the cause of disease in most instances. Further work is necessary to connect the behavior of mutant nuclear lamins and their resulting effects on nuclear envelope structure to muscular dystrophy, cardiomyopathy and lipodystrophy.
REFERENCES


