Distribution of emerin and lamins in the heart and implications for Emery–Dreifuss muscular dystrophy

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Emerin is a nuclear membrane protein which is missing or defective in Emery–Dreifuss muscular dystrophy (EDMD). It is one member of a family of lamina-associated proteins which includes LAP1, LAP2 and lamin B receptor (LBR). A panel of 16 monoclonal antibodies (mAbs) has been mapped to six specific sites throughout the emerin molecule using phage-displayed peptide libraries and has been used to localize emerin in human and rabbit heart. Several mAbs against different emerin epitopes did not recognize intercalated discs in the heart, though they recognized cardiomyocyte nuclei strongly, both at the rim and in intranuclear spots or channels. A polyclonal rabbit antiserum against emerin did recognize both nuclear membrane and intercalated discs but, after affinity purification against a pure-emerin band on a western blot, it stained only the nuclear membrane. These results would not be expected if immunostaining at intercalated discs were due to a product of the emerin gene and, therefore, cast some doubt upon the hypothesis that cardiac defects in EDMD are caused by absence of emerin from intercalated discs. Although emerin was abundant in the membranes of cardiomyocyte nuclei, it was absent from many non-myocyte cells in the heart. This distribution of emerin was similar to that of lamin A, a candidate gene for an autosomal form of EDMD. In contrast, lamin B1 was absent from cardiomyocyte nuclei, showing that lamin B1 is not essential for localization of emerin to the nuclear lamina. Lamin B1 is also almost completely absent from skeletal muscle nuclei. In EDMD, the additional absence of lamin B1 from heart and skeletal muscle nuclei which already lack emerin may offer an alternative explanation of why these tissues are particularly affected.

INTRODUCTION

The nuclear membrane protein, emerin, is the product of an X-linked gene and is either absent or defective in Emery–Dreifuss muscular dystrophy (EDMD) as a result of mutations in that gene (1–4). Emerin belongs to a family of type II integral membrane proteins which extend into the nucleoplasm from a transmembrane domain spanning the inner nuclear membrane (3). Other members of this family are lamin B receptor (LBR) (5) and two groups of lamina-associated proteins, LAP1 and LAP2 (6,7). These proteins may provide structural integrity to the inner nuclear membrane, in addition to mediating attachment between heterochromatin and the nuclear lamina. The lamina is a network of intermediate filaments lying immediately adjacent to the inner nuclear membrane and made up of lamin subunits. There are two major types of lamin: A-type (lamins A and C) and B-type (B1 and B2). Lamin A and C are two alternatively transcribed products of a primary transcript recently assigned to chromosome 1q21.3 (8). The lamin B1 and B2 genes have been localized to chromosome 5 and 19, respectively (8).

LAP2 specifically interacts with B-type lamins in a phosphorylation-dependent manner (6). LBR also interacts with B-type lamins (5) while LAP1 interacts with both A-type and B-type lamins (6). Direct interaction of emerin with lamins has not yet been demonstrated though they do co-localize in interphase cells and at certain stages of mitosis (9).

Although emerin is expressed in all tissues and cell lines tested (3), its absence in EDMD affects two tissues in particular, heart and skeletal muscle (10). Myopathic changes are evident in some skeletal muscles, and early contractures are observed at the neck, elbows and Achilles tendons (10). In the heart, conduction defects occur and often necessitate a pacemaker (10). Cartegni et al. (11) recently reported the presence of emerin at intercalated discs in...
the heart, as well as in nuclei, suggesting an attractive explanation for the cardiac conduction defects observed in EDMD. We now present results, obtained with a panel of 16 mAbs against emerin, which do not appear consistent with the hypothesis that the immunostaining of intercalated discs is due to emerin or a product of the emerin gene. As an alternative hypothesis to explain the specificity of the disease, the absence of lamin B1 from cardiac and skeletal muscle nuclei raises the possibility that emerin becomes particularly important for nuclear function in cells which lack lamin B1 and its interactions with LAP2 and lamin B receptor.

RESULTS

Monoclonal antibody characterization

All antibodies are subject to cross-reaction with other proteins, so it is important to characterize mAbs in detail and to show that they recognize different regions of the emerin molecule. We described earlier the production of 12 mAbs against the first 188 amino acids of emerin (3). A further four mAbs have now been prepared using full-length recombinant emerin as immunogen. All 16 mAbs are specific for emerin on western blots, and no mAb-specific bands are seen on blots from EDMD null mutants (3 and data not shown). The binding sites on emerin have been identified for 15 of these mAbs using phage-displayed peptide libraries and the results are shown in Figure 1. The principle of this method is that mAbs attached to solid phase select epitope-specific peptides from a library of random peptide sequences. Those amino acids in each peptide which match the antigen sequence are the important residues for mAb binding, while other amino acids in the peptide may be irrelevant to binding. Six epitopes identified in this way are shown in Figure 1, with the matching peptides aligned below the complete human emerin sequence. Six of the mAbs recognize an epitope near the N-terminus, though they differ slightly in their specificity (MANEM13 does not require Thr14). The absence of Thr14 and Leu16 in mouse and rat emerin may explain why these mAbs are human-specific. MANEM4 and 11 recognize the same epitope encoded by exon 3, while the MANEM1 and 2 epitope is at the exon 3–exon 4 junction. These epitopes are also absent from mouse and rat, though MANEM1 and 2 do recognize rabbit emerin. The rabbit sequence is not known but we would predict that Gly89 would replace the Asp89 in mouse and rat. Two new mAbs, MANEM15 and 16, were mapped to the end of the nucleoplasmic region, very close to the hydrophobic transmembrane sequence. Once again, lack of cross-reactivity with mouse and rat emerin confirms the phage display evidence that Arg221 and Gly224 are important for mAb binding. MANEM5 was unusual in recognizing peptides from two different regions of emerin and it may recognize a conformational epitope assembled by protein folding. The second part of the epitope is identical in rat emerin, so the contribution of the first part to the epitope explains why MANEM5 does not recognize rat emerin (data not shown). The emerin structure is not known, but both parts of the MANEM5 epitope are in predicted loops which could lie together on the protein surface. It is well established that secondary structures and some tertiary folding of core structures can reform on western blots after SDS treatment (cf. ref. 12). MANEM3 and 6 may also recognize this assembled surface structure, though they differ from MANEM5 in having a preference for the second and first parts of the epitope respectively (Fig. 1). Epitope mapping using phage-displayed peptides is consistent with earlier mapping of MANEM1–10 by fragmentation methods (3). Thus, MANEM7–10 and MANEM1 and 2 were placed in separate epitope groups, while MANEM3 was the only mAb not to recognize the N-terminal emerin fragment produced by cleavage at the single cysteine in the sequence.

Is emerin present at intercalated discs?

In view of reports of staining of intercalated discs in the heart by polyclonal rabbit anti-emerin antibodies (11), we were surprised to find that intercalated disc staining was absent or weak when our mAbs were used. Figure 2 shows that MANEM5 stains the nuclear membrane strongly in cardiomyocytes (Fig. 2a) but that intercalated discs, revealed by double-labelling with a rabbit anti-cadherin antibody (Fig. 2b), are not stained. The same negative result was obtained with MANEM8 at the emerin N-terminus (Fig. 2c) and with six other mAbs (MANEM2, 3, 6, 7, 10 and 14; data not shown). Faint staining of discs was observed with five mAbs (MANEM1 Fig. 2d; MANEM9, 11 13 and 16; data not shown). Only two mAbs showed clear staining of intercalated discs, MANEM4 (Fig. 2e) and MANEM15 (Fig. 2f). Nuclear membrane staining was similar in intensity for all mAbs and was much stronger than any disc staining (Fig. 2).

We were able to reproduce the observation of Cartegni et al. (11) and show that a polyclonal antiserum raised in rabbits stained both cardiomyocyte nuclei and intercalated discs (Fig. 3a and b). This result was obtained with first bleed serum, but the intercalated disc staining was much less evident with later serum bleeds from the same rabbit, as used in Figure 5 below. We also tested sera from four unimmunized rabbits and observed that three out of four sera were positive for disc staining (data not shown). When we affinity purified the serum on a pure emerin band cut from a PVDF western blot of the recombinant antigen, nuclear membrane staining was retained but intercalated disc staining was no longer evident (Fig. 3c and d), whereas the antiserum fraction which failed to bind to the emerin band still stained discs strongly (Fig. 3e). When the antiserum was affinity purified using areas of the PVDF blot either above or below the emerin band, the eluted antibodies recognized intercalated discs but not nuclei (Fig. 3f). We were not able to identify any specific protein contaminant on the blot which was absorbing the disc-binding antibodies. This experiment was performed twice using different western blots for affinity purification, and the same results were obtained.

Taken together, these data do not support the hypothesis that authentic emerin is present at intercalated discs in the heart.

Localization of emerin and lamins in the human heart

The distribution pattern for both emerin and lamin B2 in cardiomyocyte nuclei was similar (Fig. 4). Both were present at the nuclear membrane and appeared to be localized in discrete channels transecting the nucleus. A similar distribution of emerin, and co-localization with all lamin types, has been observed in interphase nuclei of cultured COS cells (9).

Figure 5 shows double-labelling of human heart with emerin (red) and various forms of lamin (green). Strong emerin staining was found in the cardiomyocyte nuclei, with weaker staining in some of the interstitial cell nuclei, whereas lamin B2 was distributed more uniformly in nearly all nuclei (Fig. 5). Higher power photographs using a dystrophin mAb to outline the myocyte membranes clearly distinguish the large myocyte nuclei
Figure 1. Identification of epitopes recognized by a panel of 16 mAbs against emerin using random peptide display libraries. The human emerin sequence of 254 amino acids is shown on five lines beginning with the amino acid number in italics. The transmembrane sequence near the C-terminus is underlined. Antibody-binding sequences obtained from peptide libraries are aligned below the emerin sequence with matching amino acids in bold face, and are followed by the mAbs which recognize them in brackets. In square brackets above, the corresponding mouse [U79753 (22)] or rat [X98377 (23)] sequences are shown in italics to show why these mAbs do not recognize mouse or rat emerins (see text). Peptides of 15 amino acids were obtained from a phage-displayed peptide library while peptides of 12 amino acids or less were obtained from peptides displayed on a bacterial flagellin protein (pFliTrx; Invitrogen).

DISCUSSION

The results presented here cast some doubt on an earlier proposal that emerin is partly localized at intercalated discs in the heart and that the cardiac conduction defect in EDMD is due to the absence of emerin from these discs. Although all 15 mAbs tested on human heart stained nuclei strongly, only two of these gave clear staining of intercalated discs, and eight mAbs showed no disc staining at all. Furthermore, the apparently random distribution of the epitopes recognized by mAbs which showed some disc staining does not support the possibility of an alternatively spliced short form of emerin at the discs, and no mRNA evidence for alternative splicing has been reported. Preferential masking of some emerin epitopes at the intercalated discs, but not at the nuclear membrane, seems unlikely but cannot be ruled out. The possibility of a protein product of an emerin-related gene sharing several epitopes with emerin cannot be ruled out, but this protein would not be affected by emerin gene mutations. The careful characterization by epitope mapping of the mAbs used in this study has added considerably to the weight of our arguments and to the value of this panel of mAbs for future EDMD research. The demonstration of a conformational epitope has also provided the first experimental evidence for protein folding and tertiary structure in the emerin molecule.

Although we were able to reproduce the observation of Cartegni et al. (11) that a rabbit antiserum against emerin stains both nuclei and intercalated discs, we were also able to separate nuclear staining from disc staining by affinity purification. Nuclear staining antibodies were absorbed specifically by the emerin band on a western blot, but absorption of disc-staining antibodies could not be attributed to any single contaminant protein in the recombinant emerin preparation nor to any particular region of the western blot. It seems unlikely, therefore, that we will be able to resolve this issue conclusively by identifying a non-emerin protein responsible for the disc staining. Non-specific staining at intercalated discs is supported further by the fact that three out of four pre-immune rabbit sera stained discs quite strongly and the fact that, after persistent booster immunizations, disc staining tended to disappear from our rabbit antiserum while nuclear
Figure 2. Localization of emerin in human heart sections using a panel of mAbs. Double-labelling with MANEM5 (a) and rabbit anti-PAN-cadherin antibody (b) (Sigma; 1/100) shows that intercalated discs (arrows in b) are not stained by the emerin mAb, though seven or eight large cardiomyocyte nuclei in (a) are strongly stained. MANEM8 is another example of no disc staining (c), while MANEM1 illustrates the weak staining of discs displayed by some mAbs (d). MANEM4 (e) and MANEM15 (f) showed clear and consistent staining of discs (arrows), though it is still less bright than the nuclear staining.

staining became stronger. It should be noted that Cartegni et al. (11) used a rabbit which gave no pre-immune disc staining and that disc staining was not removed from their antiserum by affinity purification.

A conclusive test of the intercalated disc hypothesis could be performed using heart tissue from a null mutant in the emerin gene, since any non-specific staining, or emerin-related gene product, at intercalated discs would be expected to remain while authentic emerin would be absent. Unfortunately, heart tissue from EDMD patients currently is not available for research and an emerin knockout mouse has not yet been produced. Detectable conduction defects may take many years to develop in EDMD, so it seems unlikely that emerin can play a direct or essential role in cardiac conduction. They may instead be secondary defects resulting from rather specific degenerative changes, similar to those which evidently occur in certain skeletal muscles in EDMD.

In view of these findings, we are forced to consider how the absence of emerin from the nuclear membrane in EDMD might account for the specificity of the disease for skeletal and cardiac muscles. The observation that some nuclei contain emerin while others do not was first made in a variety of tissues by Nagano et al. in 1996 (2), though the cell types in the heart which lack emerin were not defined in this study. Surprisingly, the observation that lamins also show a cell-specific distribution in tissues was not clearly made until 1997 (13). Current ideas about translocation of nuclear membrane proteins, like LAP2 and LBR, is that they are inserted into the endoplasmic reticulum where they diffuse laterally until they are trapped in the nucleus by their interaction with lamins (15). If emerin is translocated in a similar way, then lamin B1 is clearly not essential for trapping emerin because it is absent from most emerin-positive cells in the heart (Fig. 5). The sequence in LAP2 involved in lamin B1 binding has been
identified (16) and has no similarity to the emerin sequence. Although the distribution of lamin A is most similar to emerin in the heart, any of the non-B1 lamins could be involved in trapping emerin. Indeed, since direct interaction between emerin and lamins has not yet been demonstrated, a non-lamin protein might be involved instead. However, these considerations do raise the possibility that the function of emerin in the nuclear membrane is different from, and possibly complementary to, that of the system of B-type lamin plus LAP2/LBR. In EDMD, the viability of cardiomyocytes and skeletal muscle fibres may be compromised by their lack of both lamin B1 and emerin and this may account for the specificity of the disease for these tissues.

The observation that emerin distribution most closely resembles that of lamin A is of particular interest since lamin A is a candidate gene for the less common, autosomal form of EDMD which is different from, and possibly complementary to, that of the system of B-type lamin plus LAP2/LBR. In EDMD, the viability of cardiomyocytes and skeletal muscle fibres may be compromised by their lack of both lamin B1 and emerin and this may account for the specificity of the disease for these tissues.

MATERIALS AND METHODS

Cloning and expression of full-length emerin cDNA

Emerin cDNA was cut with StyI and Ddel, producing a blunt-ended fragment which was ligated into pET17b cut with BsoXI to create an in-frame construct expressing full-length emerin protein in Escherichia coli BL21(DE3). The recombinant protein obtained after induction with isopropyl-β-D-thiogalactopyranoside (IPTG) was partially purified from inclusion bodies by sequential extraction with increasing concentrations of urea in phosphate-buffered saline (PBS). Protein extracted with 2 M urea was used for immunization.

Production of antibodies

mAbs were produced by immunization of BALB/c mice with recombinant emerin and fusion of spleen cells with Sp2/0 myeloma cells as described elsewhere (18). Hybridoma supernatants were tested for emerin specificity by enzyme-linked immunosorbent assay (ELISA), western blotting and immunofluorescence microscopy, and four new cell lines were successfully established after two rounds of cloning by limiting dilution.

A rabbit polyclonal antiserum was produced by immunization at monthly intervals of a New Zealand White rabbit with 1 mg of a recombinant protein containing the first 188 amino acids of emerin (3) in Freund’s adjuvant. Serum samples were taken 10 days after the second and subsequent injections.

Epitope mapping

Selection of peptides from random libraries was performed by reiterative bio-panning of phage or E.coli against mixtures of mAbs captured on a Petri dish with rabbit anti-mouse Ig (Dako) as described previously (19). The 15mer libraries displayed on the pIII coat protein of filamentous bacteriophage fuse5 were provided by Professor George P. Smith (University of Missouri). The 12mer libraries displayed within a thioredoxin insert on E.coli flagellin were obtained as a FlTrx kit and were used according to the manufacturer’s instructions (Invitrogen). Phage or plasmid sequences expressing the peptide were determined by DNA sequencing with Sequenase v.2.0 (Amersham). With some mAbs, many positive phage clones (10–20) were obtained from the libraries but only two or three were sequenced in order to identify the epitope location; identical peptide sequences were often found in more than one phage clone. Sometimes peptide sequences were obtained which bore no obvious similarity to the emerin sequence, but this was uncommon.

Immunofluorescence microscopy

Unfixed, frozen sections of human and rabbit tissues (5 μm) were mounted on glass slides and stored at −70°C. Fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG (Dako) was used to detect bound antibody as described previously (20). For double-label experiments, sections were incubated with a mixture of rabbit anti-emerin serum (1/100) and one mouse anti-lamin mAb for 30 min followed by a mixture of tetramethylrhodamine isothiocyanate (TRITC)-labelled swine anti-rabbit Ig (1/20; Dako) and FITC-labelled horse anti-mouse Ig (1/20; Vector) for 30 min. The lamin mAbs were: lamin B2 (1:50 dilution; Novocastra Laboratories, Newcastle upon Tyne, UK), lamin B1 (1:50; Chemicon, Temecula, CA), lamin A (1:5; Chemicon) and lamin A/C (1:200; Chemicon). Images were obtained using a Leica DMILB microscope with 10x, fluotar 40x or 100x objectives, and overlays were produced by computer merging after capture.

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Figure 5. Double-labelling of human heart sections with rabbit anti-emerin serum and mAbs against various laminas. Double-labelling using unpurified rabbit anti-emerin serum (first column; sixth bleed; 1/100) and various anti-lamin mAbs (third column; see Materials and Methods). Co-localization is usually revealed by orange or yellow labelling in the overlay of the two images (middle column) but this should also be checked by direct comparison of the first (TRITC) and third (FITC) images. In the higher power images of lamin B2 (second row), a dystrophin mAb [MANDYS1 (21); 1/50] was added with the lamin mAb to outline the cardiomyocyte membranes and to show that smaller nuclei with lamin B2 and no emerin lie outside the myocytes.
REFERENCES


