Heart-specific localization of emerin: new insights into Emery–Dreifuss muscular dystrophy

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Emery–Dreifuss muscular dystrophy (EDMD) is an X-linked inherited disease characterized by early contracture of the elbows, Achilles tendons and post-cervical muscles, slow progressive muscle wasting and weakness and cardiomyopathy presenting with arrhythmia and atrial paralysis: heart block can eventually lead to sudden death. The EDMD gene encodes a novel ubiquitous protein, emerin, which decorates the nuclear rim of many cell types. Amino acid sequence homology and cellular localization suggested that emerin is a member of the nuclear lamina-associated protein family. These findings did not explain the role of emerin nor account for the skeletal muscle- and heart-specific clinical manifestations associated with the disorder. Now we report that emerin localizes to the inner nuclear membrane, via its hydrophobic C-terminal domain, but that in heart and cultured cardiomyocytes it is also associated with the intercalated discs. We propose a general role for emerin in membrane anchorage to the cytoskeleton. In the nuclear envelope emerin plays a ubiquitous and dispensable role in association of the nuclear membrane with the lamina. In heart its specific localization to desmosomes and fasciae adherentes could account for the characteristic conduction defects described in patients.

INTRODUCTION

Emery–Dreifuss muscular dystrophy (EDMD) is an X-linked muscular disease first described in the early 1960s and characterized by early contracture of the elbows, Achilles tendons and post-cervical muscles and slow progressive muscle wasting and weakness, with humeroperoneal distribution in the early stages of the disease, and cardiomyopathy usually presenting as a heart block (1). The cardiomyopathy manifests as a cardiac conduction defect and the associated heart block is a frequent cause of death. Provided that diagnosis is made sufficiently early, the insertion of a cardiac pacemaker can be life saving. In no case has mental retardation, intellectual defect or involvement of other organs been described.

The EDMD gene was identified among a large number of candidates in a very gene rich region where the disease had been mapped (2). The EDMD gene is ubiquitously expressed and encodes a novel ubiquitous protein of 254 amino acids, emerin, localized at the nuclear rim of most cell types analyzed (2–5). Emerin showed limited sequence similarity with LAP2, a ubiquitous integral membrane protein involved in association between the nuclear lamina and nuclear envelope (6). The similarities between the two proteins suggested that emerin is a member of the nuclear lamina-associated protein family (6). This finding was quite unique, as most muscle or cardiac disorders are caused by alterations in molecules specifically or highly expressed in muscle tissues.

We have further investigated emerin localization and we have conclusively established that emerin is indeed an integral membrane protein of the inner nuclear membrane. In this paper we report that emerin localizes to the inner nuclear membrane, via its hydrophobic C-terminal domain, but that in heart and cultured cardiomyocytes it is also associated with the intercalated discs. We propose a general role for emerin in membrane anchorage to the cytoskeleton. In the nuclear envelope emerin plays a ubiquitous and dispensable role in association of the nuclear membrane with the lamina. In heart its specific localization to desmosomes and fasciae adherentes could account for the characteristic conduction defects described in patients.

RESULTS

Emerin is a phosphorylated protein

From the sequence analysis emerin appeared to possess multiple phosphorylation sites for a range of kinases (2). In vivo labeling of HeLa cells with [32P]orthophosphate confirmed that emerin is phosphorylated in vivo (Fig. 1a). The antibodies immunoprecipitated two phosphorylated bands of 34 and 36 kDa of the same size as those commonly seen in Western blots. Treatment of

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HeLa cells with the phosphatase inhibitor okadaic acid (7) caused a relative increase in the 36 kDa form over the more abundant 34 kDa form and demonstrated that the two forms correspond to different levels of phosphorylation of emerin (Fig. 1b).

The C-terminal hydrophobic domain of emerin is the determinant for nuclear membrane localization

Emerin is a small hydrophilic protein with a hydrophobic tail of 21 amino acids, 11 residues from the C-terminus. To establish whether the C-terminal hydrophobic domain of emerin was important for nuclear rim localization, mutants were constructed carrying different short deletions spanning the entire protein and an HA epitope tag at the N-terminus (Fig. 1c). Upon transfection into COS cells the wild-type protein localized to the nuclear envelope (Fig. 1d), as did proteins lacking the conserved region at the N-terminus (2) or with any of the internal deletions. Only the mutant lacking the last 27 amino acids spanning the C-terminal putative transmembrane domain (HA–ΔTrans) was unable to localize to the nuclear envelope and was concentrated inside the nuclei (Fig. 1d). To confirm that the hydrophobic domain is essential for membrane localization, the transmembrane domain was fused to the C-terminus of the reporter protein GFP (GFP–Trans). The 27 C-terminal amino acids were sufficient to drive most of the transfected chimeric protein to the nuclear periphery (Fig. 1d).

Emerin is localized to the inner nuclear membrane

Electron microscopy immunogold studies of ultrathin frozen sections of skeletal muscle, heart and HeLa cells showed that emerin is associated with the inner nuclear membrane. In skeletal muscle sections most gold granules localized to the nuclear lamina or the inner nuclear membrane (Fig. 1) at positions where the two membranes are well separated by enlargements of the intranuclear cisterna. Similar results were obtained in heart (Fig. 1f) and HeLa cells (not shown).

Nuclear localization is insufficient for emerin function

Emerin appears to be an integral membrane protein localized in the inner nuclear membrane and the main determinant of this localization resides in the C-terminal hydrophobic stretch, which can be considered a genuine transmembrane domain. A nuclear localization is, however, insufficient to ensure a normal phenotype and the stability and/or the specificity of emerin binding to other proteins must involve determinants in the rest of the protein. This is demonstrated by the behavior of a mutated emerin found in one patient (LB1520) presenting a frameshift mutation in the C-terminal moiety (2). In this patient a shorter protein was predicted, deleted of the emerin sequence starting from amino acid 169 but possessing a new hydrophilic tail compatible in amino acid composition and length with a nuclear envelope transmembrane domain (8; Fig. 2a). Such a shorter emerin was indeed synthesized (5) and immunohistochemical analysis of a muscle biopsy of the patient demonstrated that a reduced but still detectable amount of emerin was at the nuclear rim (Fig. 2b).

Subcellular distribution of emerin

As previously shown by Manilal et al. (4), in brain tissue emerin was not exclusively found in the nuclear envelope. Cell fractionation of several cell types by mechanical shearing in hypotonic buffer separated emerin into nuclear and cytoplasmic ‘pools’. In HeLa cells (Fig. 3a) the nuclear emerin (lane 2) was found in the lamina-associated fraction, resistant to nuclease digestion, high salt and detergent (lane 10). The cytoplasmic emerin (lane 3) co-localized with membranes (lane 4) but, unlike that in the nuclear fraction, it was readily solubilized in the presence of detergents (lines 5 and 6). Correct fractionation of lamin A in nuclei and vimentin in cytoplasm excluded major cross-contamination between the fractions. The relative abundance of emerin in the cytoplasmic membrane-associated fraction varied slightly in different cell types: fibroblasts, myoblasts, HepG2 and neuroblastoma cells were analyzed and examples are shown in Figure 3b. No great differences were found in myoblasts compared with myotubes (not shown) and between human, rat and simian cells, though a smaller amount of cytoplasmic emerin was usually found in myoblasts, especially from rat.

Similar results were obtained by fractionation of human heart tissue, using a slightly different protocol to separate nuclear and cellular membranes (Fig. 3c) from soluble proteins. The cytoplasmic emerin was found with the cadherins, in cytoplasmic membranes (Fig. 3c, lane 4).

An alternative localization of emerin in heart

Immunohistochemical analysis of cells and tissues did not show any significant association of emerin with specific cytoplasmic structures. The only notable exception was the heart. In unfixed cryosections of human heart the antibodies also decorated heart intercalated discs (Fig. 4a). Three different antisera, two raised against the human (5) and one against the mouse protein, gave identical results in human, mouse and rat heart. Identical results were obtained with the antisera and with affinity-purified antibodies. No reaction was observed with preimmune serum and all labeling was titrated away when excess recombinant antigen

Figure 1. (a) Immunoprecipitation of emerin from metabolically 32P-labeled HeLa cells (18) was performed using anti-emerin antibodies (D7) or pre-immune serum (C). Phosphorylated emerin was visualized by autoradiography following separation by 10% SDS–PAGE. (b) Western blot analysis of emerin in mock-treated cells (–OA) or cells harvested after 90 min exposure to 1 μM okadaic acid (+OA). (c) Schematic representation of the emerin deletion mutants and their localization in COS cells (Loc). A schematic representation of emerin is shown at the top. The black box represents the hydrophobic domain, gray boxes represent serine-rich regions and open slashed boxes represent LAP homologies. Constructs contained the HA epitope (rightward slashed box) or GFP (oval) fused to the indicated portions of emerin. N.E., nuclear envelope; N, nucleus; NC, nucleus and cytoplasm. Experiments depicted in (d) are underlined. (d) Immunohistochemical localization of some of the fusion proteins. The HA fusions (HA–wt and HA–ΔTrans) were analyzed by indirect immunofluorescence on methanol-fixed cells with anti-HA antibodies. GFP fusions (GFP and GFP–Trans) were analyzed by direct fluorescence as recommended by the manufacturer. (e) Section through a human skeletal muscle. The gold granules are specifically localized along the inner nuclear membrane, corresponding to the nuclear lamina. The outer nuclear membrane (arrow) is not as well labeled as the nucleoplasm (N) or the myofilaments (M). x75000. (f) Section through a human cardiomyocyte nucleus. The gold granules are mostly localized along the inner nuclear membrane. The cutting plane is sometimes not perpendicular to the nuclear envelope in correspondence with the nuclear indentation. This causes an apparent shift of the gold granule images with respect to the nuclear envelope image. The gold granules, however, follow the external borders of the peripheral heterochromatin, strongly suggesting an association with the nuclear lamina (arrowheads). Few gold granules are visible over the cytoplasm. x46000.
was added to the incubation (not shown). Serial 6 µm thick sections of an entire adult mouse heart demonstrated that emerin stained nuclei and intercalated discs throughout the organ (not shown).

Primary cultures of cardiomyocytes prepared from 21 d.p.c. rat fetuses (9) were also studied. After 2–3 days in culture adjacent cardiomyocytes formed intercalated discs: in such culture conditions we could confirm emerin localization at the nuclear rim of cardiomyocytes and fibroblasts and on newly formed intercalated discs (Fig. 4b). Confocal analysis of the cultures double stained with anti-emerin and anti-cadherin or anti-vinculin antibodies demonstrated a third site of emerin localization at the base of the cardiomyocytes. The striking similarity between emerin and vinculin distribution, although only in part superimposable, indicated that both mark focal adhesions (10,11). Vinculin, however, also marked focal adhesions of flanking fibroblasts, whilst emerin in focal adhesions was specific for cultured cardiomyocytes.

Finally, electron microscopic immunogold cytochemistry of ultrathin frozen sections of human heart confirmed that emerin is part of the adhesive junctions of the heart. The gold granules were localized around desmosomes and fasciae adherentes (Fig. 5a and b), at the periphery of the dense fibrillar material. The regions between adhesion structures (Fig. 5c) and gap junctions (not shown) were not labeled.

**DISCUSSION**

The data presented in this paper confirm the suggestion that emerin, the product of the X-linked EDMD gene is a ubiquitous highly phosphorylated integral membrane protein of the nuclear envelope, tightly associated with the nuclear lamina. As predicted, emerin is indeed phosphorylated in vivo and the different phosphorylated forms may play important roles in interactions with the nuclear membrane and other nuclear proteins, during the cell cycle or in different cell types (12,13). Our data showed that the main determinant for inner nuclear membrane targeting is the C-terminal hydrophobic domain, which can be considered a genuine transmembrane domain. In the absence of the C-terminal domain in transfected COS cells most of the protein was localized in the nucleus, suggesting that a nuclear localization signal exists in the remaining portion of the protein. In vivo synthesis of a mutated emerin having a new C-terminal hydrophobic domain with an amino acid composition compatible with nuclear membrane insertion was sufficient for nuclear envelope localization.

In many cell types and tissues subcellular fractionation experiments demonstrated that a relevant fraction of emerin is also associated with cytoplasmic membranes. The finding of this fraction in tissues (see Results; 4) as well as in dividing cells indicates that it may not merely represent newly synthesized emerin on its way to the nuclear membrane and suggests different and possibly specific roles for emerin. In heart the cytoplasmic membrane-associated fraction appears to be part of the highly specialized heart adhesive junctions, desmosomes and fasciae adherentes of the intercalated discs. The evidence came primarily from immunohistochemical analysis using three different affinity-purified antibodies. Two were previously described (5), raised against a human protein lacking the C-terminal domain (amino acids 1–168). The third was raised in rabbits against a similar protein (amino acids 1–168) deduced from a mouse emerin cDNA sequence presenting 75% amino acid identity to the human sequence (Cartegni et al., in preparation). In Western blots the three antibodies reacted almost exclusively with emerin and did not show heart-specific bands. This result was confirmed by electron microscopic analysis of heart sections, by immunohistochemistry of cardiomyocyte cultures and subcellular fractionation experiments. Moreover, confocal analysis of cardiomyocyte cultures demonstrated that cardiac emerin and not emerin from fibroblasts could be recruited to newly formed focal adhesions, strongly indicating that emerin is part of a heart-specific adhesion complex. Nagano et al. (3) had previously shown that one of their polyclonal antibodies raised against a synthetic peptide (ED1, amino acids
did not have samples from patient hearts to study, but we never observed staining of the sarcolemma or of the cardiac cell outer membrane with our antibodies, which specifically stained only intercalated discs. The two reagents are, however, also very different, as both the antibodies raised by Nagano et al. were against C-terminal peptide sequences not contained in our antigen and may not recognize the same epitope(s).

Desmosomes and fascia adherentes anchor desmin-containing intermediate filaments and the bundles of sarcomeric myofilaments respectively (10). They consist of transmembrane adhesive glycoproteins, members of the cadherin superfamily, and of cytoplasmic proteins such as vinculin, catenins and actin binding proteins (10). Different assortments of the same or similar proteins in desmosomes, fascia adherentes, focal adhesions and other adhesive junctions seem to confer specific functions to ensure cell–cell communication and tight adhesion between cells and to the extracellular matrix. The role of this complex assortment of proteins is best demonstrated by the existence of many genetic diseases that perturb adhesion and in heart by the dramatic consequences of plakoglobin (γ-catenin) knock out (14); plakoglobin –/– mice die at mid-gestation due to rupture of the ventricles.

The cardiac conduction defect in EDMD patients is the most severe and life threatening clinical manifestation of the disease. Cardiac alterations have also been described in female carriers (1) in the absence of any skeletal muscle abnormality, suggesting a prominent role in cardiac conduction for emerin. Localization of emerin in heart adhesive junctions suggests lack of emerin in heart may have a direct effect on cardiac conduction abnormalities. We propose that lack or a decreased amount of emerin in heart alters cardiomyocyte adhesion and/or communication between adjacent cells and is responsible for arrhythmia and slow pacing and eventually heart block. Whether this is due to a general failure in cardiac conduction or to a more specific effect at the level of the heart pacemaker cannot be established with the present knowledge of the disease. On the other hand, in favor of our general model is the notion that emerin, with myotonic dystrophy kinase (15), is the most relevant function of this protein in both skeletal muscle and heart.

We propose that lack or a decreased amount of emerin in heart alters cardiomyocyte adhesion and/or communication between adjacent cells and is responsible for arrhythmia and slow pacing and eventually heart block. Whether this is due to a general failure in cardiac conduction or to a more specific effect at the level of the heart pacemaker cannot be established with the present knowledge of the disease. On the other hand, in favor of our general model is the notion that emerin, with myotonic dystrophy kinase (15), is the second protein localized to the intercalated discs and responsible for a genetic disorder presenting a severe heart conduction defect.

Our results suggest that emerin may associate directly with membranes through its hydrophobic tail and we expect that it may do the same at the intercalated discs. Its cytoplasmic hydrophilic portion should interact with protein(s) of the adhesive junctions. In the emerin sequence a serine-rich stretch of 12 amino acids (PV/SAR/SSL/DLS), 42 amino acids from the transmembrane domain, presented striking similarity to the consensus sequence (PV/ICFSRXSSL/SLS/DLS) of the 20 amino acid repeats of the adenomatous polyposis coli (APC) tumor suppressor gene (16), involved in binding β-catenin. It also showed significant though lower similarity to the minimal β-catenin binding region of the cadherins (17). Thus emerin in heart may also bind β-catenin or a novel protein presenting similar sequence. It is noteworthy that this part of the protein is deleted in patient LB1520, who still retains some emerin capable of correct localization.

We have no evidence yet of a specific emerin localization in skeletal muscle that may account for the muscular dystrophy of EDMD patients. The finding of emerin in cytoplasmic membranes suggests a general and novel role for emerin in membrane attachment to the cytoskeleton and this could actually represent the most relevant function of this protein in both skeletal muscle and heart.

Figure 3. Western blot analysis of emerin in cell fractions. (a) Subcellular fractionation of HeLa cells. Fractions were prepared as described in Materials and Methods and probed with anti-emerin, anti-vimentin, anti-lamin B and anti-lamin A/C antibodies. Lane 1, total extract; lane 2, nuclei; lane 3, cytoplasm; lane 4, high speed cytoplasmic pellet; lane 5, 1% Triton X-100 cytoplasmic pellet wash; lane 6, detergent-resistant cytoplasmic pellet; lane 7, high speed cytoplasmic supernatant; lane 8, high speed cytoplasmic supernatant wash; lane 9, 1% Triton X-100 cytoplasmic supernatant; lane 10, 1 M NaCl wash; lane 11, 1% Triton X-100 wash; lane 12, detergent-resistant cytoplasmic supernatant; lane 13, detergent-resistant cytoplasmic supernatant wash; lane 14, immunoprecipitation with monoclonal antibodies against C-terminal peptide sequences not contained in our antigen and may not recognize the same epitope(s).
Figure 4. Emerin localization in heart. (a) Indirect immunofluorescence analysis of 6 µm thick human heart cryosections, double stained with antibodies to the indicated proteins. Emerin gives signals on the nuclear rim (arrowhead) and on intercalated discs (arrow). Lamin B stains nuclei; vinculin stains intercalated discs and sarcolemmal membranes; cadherin stains intercalated discs. (b) Emerin localization in cultured fetal rat cardiomyocytes, methanol-fixed 72 h after plating and analyzed by confocal microscopy using a BioRad MRC-1024 confocal microscope equipped with a Kr/Ar laser. Emerin is shown in green, cadherin and vinculin in red. The two images were superimposed to show co-localization (yellow) at the intercalated discs (emerin + cadherin and emerin + vinculin) and at focal adhesions (emerin + vinculin). Arrows indicate the intercalated discs; arrowheads indicate fibroblasts; emerin exclusively stains the nuclei and vinculin the focal adhesions. Focal adhesions are positive to emerin only in cardiomyocytes. The lower panel shows a detail of the intercalated disc between twofour three cardiomyocytes.
Heart tissue was processed as described by Kuehl et al. (19) with minor modifications. A crude homogenate of 500 mg human heart in 25 mM Tris–HCl, pH 7.1, 2.5 mM MgCl₂, 0.25 M sucrose, 0.6% (w/v) Triton X-100 and protease inhibitors was centrifuged at low speed. The cellular pellet was resuspended in 25 mM Tris–HCl, pH 7.1, 2.5 mM MgCl₂, 2.3 M sucrose and protease inhibitors and centrifuged for 75 min at 82 000 g through a 2.3 M sucrose cushion to separate the soluble cytoplasmic fraction, an insoluble fraction which accumulated at the interface and a pellet containing purified nuclei. These were treated as above to obtain the nuclear lamina-associated fraction.

**Production of antisera**

The anti-human emerin antisera have been described previously (5). Rabbit polyclonal antisera were raised against a bacterial fusion protein expressing a fragment of mouse recombinant emerin corresponding to amino acids 1–168. The mouse sequence was deduced from a mouse cDNA obtained in the laboratory (Cartegni et al., in preparation) and the PCR amplification product was cloned in vector pGEX 2T and controlled by sequencing as described for the human cDNA. Specific antibodies were isolated from the sera by affinity purification using antigen immobilized on nitrocellulose filters (20).

**Immunocytochemistry and Western blots**

Indirect immunofluorescence and Western blots were performed as described previously (5), with the anti-emerin antibodies used at 1:100 or 1:1000 dilutions respectively. The 12CA5 mouse anti-HA mAb (Boehringer), the anti-vimentin, anti-vinculin and anti-cadherin (CH-19) mAb (Sigma, St Louis, MO) and the mouse anti-lamin A/C mAb (Ylem, Avezzano, Italy) were used as indicated by the suppliers.

Electron microscopy analysis was performed as described previously (21) on normal skeletal muscle samples obtained from a non-dystrophic patient during surgical treatment of osteosarcoma or heart samples taken from routine control biopsies on a heart transplant patient. The tissue fragments were fixed with 0.001% glutaraldehyde, 2% paraformaldehyde in 0.1 M NaPO₄ buffer, pH 7.2, for 1 h at 4°C, cryoprotected in 2.3 M sucrose overnight at 4°C, frozen in liquid nitrogen and sectioned with a Reichert FC4 cryosectioning unit at –95°C (21). The anti-emerin antibodies were used diluted 1:50. Cryosectioning was the only method among several tested that allowed emerin immunostaining.

**Constructs and transfections**

Emerin deletion mutants were prepared by PCR, inserted in-frame in a vector containing the HA epitope, sequenced and cloned in pMT2 (22). Alternatively, PCR-amplified fragments of emerin were cloned in vector pEGFP-C1 (Clontech). COS cells were transiently transfected by the standard CaPO₄ method and analyzed 24 h after transfection.

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