ORIGINAL ARTICLE

Lamin A N-terminal phosphorylation is associated with myoblast activation: impairment in Emery–Dreifuss muscular dystrophy

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Lamins A and C are different splicing products of the *LMNA* gene. Both proteins are located at the nuclear lamina and interact with various nuclear envelope and nuclear matrix constituents including emerin, lamin B, LAP2 α , pRB, nuclear actin, and AKAP149.¹ Lamin A is also localised in splicing factor compartments inside the nucleus,² where it is involved in mRNA processing. Moreover, the carboxy-terminal domain of lamin A/C mediates an interaction with chromatin which occurs in a non-sequence-specific manner.³

Intermolecular interactions of lamin A/C are likely to be regulated in a tissue specific and possibly function related fashion. This is strongly suggested by the increasing number of diseases associated with *LMNA* gene mutations. To date, eight different disorders have been linked to lamin A/C mutations, including autosomal dominant and autosomal recessive Emery–Dreifuss muscular dystrophy (EDMD2 and EDMD3, respectively),^{4–5} limb girdle muscular dystrophy 1B,⁶ dilated cardiomyopathy with conduction defects,⁷ Charcot–Marie–Tooth axonal neuropathy type 2,⁸ familial partial lipodystrophy,⁹ mandibuloacral dysplasia,¹⁰ Hutchinson–Gilford progeria syndrome, and atypical forms of Werner syndrome.^{11–14} In some instances these diseases share clinical features, but each is characterised by a phenotype suggesting tissue specific defects of lamin A/C function. Emery–Dreifuss muscular dystrophy is characterised by muscle wasting and weakness, contractures of the elbows, Achilles tendons and neck, and dilated cardiomyopathy associated with

Background: Skeletal muscle disorders associated with mutations of lamin A/C gene include autosomal Emery–Dreifuss muscular dystrophy and limb girdle muscular dystrophy 1B. The pathogenic mechanism underlying these diseases is unknown. Recent data suggest an impairment of signalling mechanisms as a possible cause of muscle malfunction. A molecular complex in muscle cells formed by lamin A/C, emerin, and nuclear actin has been identified. The stability of this protein complex appears to be related to phosphorylation mechanisms.

Objective: To analyse lamin A/C phosphorylation in control and laminopathic muscle cells.

Methods: Lamin A/C N-terminal phosphorylation was determined in cultured mouse myoblasts using a specific antibody. Insulin treatment of serum starved myoblast cultures was carried out to evaluate involvement of insulin signalling in the phosphorylation pathway. Screening of four Emery–Dreifuss and one limb girdle muscular dystrophy 1B cases was undertaken to investigate lamin A/C phosphorylation in both cultured myoblasts and mature muscle fibres.

Results: Phosphorylation of lamin A was observed during myoblast differentiation or proliferation, along with reduced lamin A/C phosphorylation in quiescent myoblasts. Lamin A N-terminus phosphorylation was induced by an insulin stimulus, which conversely did not affect lamin C phosphorylation. Lamin A/C was also hyperphosphorylated in mature muscle, mostly in regenerating fibres. Lamin A/C phosphorylation was strikingly reduced in laminopathic myoblasts and muscle fibres, while it was preserved in interstitial fibroblasts.

Conclusions: Altered lamin A/C interplay with a muscle specific phosphorylation partner might be involved in the pathogenic mechanism of Emery–Dreifuss muscular dystrophy and limb girdle muscular dystrophy 1B.

conduction defects.¹⁵ Thus in this disorder muscle involvement is predominant, though cases presenting with lipodystrophy have also been described.¹⁶ The pathogenesis of EDMD2 has been extensively investigated in recent years,^{4–17–24} and there is circumstantial evidence that lamin A/C interaction with either nuclear envelope constituents or chromatin may be affected in the disease. However, the precise pathogenic mechanisms in EDMD2 remain unknown.

In this study, we evaluated the possibility that lamin A/C behaviour could be regulated by signalling mechanisms that cause post-translational modifications of protein and lead to different intermolecular interactions. Known post-translational modifications of lamin A/C are farnesylation, cleavage of the CaaX terminus by ZMPSTE24²⁵ and possibly by other unknown endoproteases, and phosphorylation. Lamin phosphorylation is a key event in the mitotic breakdown of the nuclear lamina.²⁶ In cells undergoing mitosis, cyclin dependent kinases mediate phosphorylation of lamins A, B, and C.²⁷ This event follows the interphase phosphorylation of lamins by protein kinase C.²⁷ Interphase phosphorylation of lamins A and C has been reported following insulin treatment of quiescent fibroblasts.²⁸ The investigators suggested that a direct mechanism may cause lamin phosphorylation, as nanomolar concentrations of insulin can elicit lamin A/C labelling.²⁸ More recently, Martelli *et al*²⁹

Abbreviations: EDMD, Emery–Dreifuss muscular dystrophy; LGMD 1B, limb girdle muscular dystrophy 1B

characterised the site of molecular interaction between lamin A and protein kinase Cα in the lamin A domain downstream of amino acid 499. Modulation of lamin A/C phosphorylation has been also reported in cells infected by murine cytomegalovirus.^{30–31} However, neither the extent nor the role of lamin A/C phosphorylation has been determined in interphase cells under physiological conditions.

In this study, we took advantage of a monoclonal antibody raised against the amino-terminus of lamins A and C which specifically binds to a phosphorylated epitope.³⁰ Our results show that lamin phosphorylation is decreased in quiescent myoblasts to almost undetectable levels, while a high level of phosphorylated lamin A can be detected in cycling myoblasts and differentiating myotubes. Lamin phosphorylation is still detectable in mature human muscle nuclei and it is increased in regenerating fibres, suggesting a role of lamin post-translational modification in muscle differentiation. Regulation of lamin A phosphorylation involves insulin signalling in both cycling and differentiated myoblasts. On the basis of these findings, we evaluated the extent of lamin phosphorylation in cultured myoblasts and muscle biopsies from EDMD2 patients. Our results show that phosphorylation of lamin A/C is dramatically reduced in EDMD2 and LGMD 1B muscle.

METHODS

Cell cultures and tissue processing

Muscle biopsies from control and laminopathic subjects were obtained after informed consent from the patients and approval of the local ethics commission. Lamin A/C mutations reported in this study have been published previously (table 1). EDMD patient 2 harboured two mutated nucleotides in the same allele, c.569G→A (p.R190Q) and c.746G→A (p.R249Q). R249Q alone was previously reported to lead to EDMD,³² while R190 was reported to be mutated in tryptophane (W) in cases of dilated cardiomyopathy with conduction defects.³³

Muscle samples were in part frozen in isopentane as previously described³⁴ for cryosectioning, and in part employed to obtain primary myoblast cultures. Cell cultures were carried out using enzymatic and mechanical treatment of muscle biopsies and plating in Dulbecco's modified Eagle's medium (D-MEM) plus 20% fetal calf serum (FCS) and antibiotics. Myotubes were obtained by confluent myoblast cultures allowed to differentiate for seven days in culture medium.

C2C12 mouse myoblasts were purchased from the European Cell Culture Collection and were cultured in D-MEM plus 10% FCS. Differentiation of myotubes was obtained by shifting subconfluent cultures in differentiation medium (D-MEM plus 1% FCS). Reserve cells were separated from myotubes (two or four days in differentiation medium), as previously reported.³⁶ Briefly, separation was obtained by trypsin detachment of cultured cells and replating in the growth medium for 30 minutes at 37°C. Myotubes were obtained in the culture medium, while the mononucleated

Figure 1 SW2-30 antiphosphorylated lamin A/C antibody specifically recognises a phosphorylated epitope. C2C12 myoblast lysates treated or not with alkaline phosphatase (100 U/ml) were subjected to western blot analysis by SW2-30 monoclonal antibody or antilamin A/C polyclonal antibody. Phosphorylated proteins (p-lamin A and p-lamin C) were only detected in untreated myoblasts (control). Antilamin A/C antibody labelled two lamin A bands, the upper corresponding to hyperphosphorylated lamin A. This band was detectable after phosphatase treatment, but the low molecular weight band showed intense staining. Phosphorylated lamin C band (p-lamin C) showed less intense staining and antilamin A/C antibody sharply labelled a lower molecular weight band, while the upper lamin C band was faintly detectable. Sodium orthovanadate was added to a control sample before alkaline phosphatase treatment to show specificity of the reaction (right lane).

resting cells attached to the plastic surface and were immediately retrieved by trypsin treatment.

Immunochemical analysis

SW2-30 antibody³⁰ is raised against lamin A/C N-terminus and the epitope recognised by the antibody appears to lie between amino acid residues 1 and 417 of the phosphorylated protein.³⁰ The specificity of SW2-30 antibody against phosphorylated lamin A/C in myoblasts was checked by treatment of C2C12 myoblast lysates with alkaline phosphatase (10 to 100 units per ml) to obtain a negative control for western blot analysis. A further control experiment using sodium orthovanadate (1 mM), which inhibits alkaline phosphatase activity, was also carried out. Western blot analysis of cellular lysates from C2C12 myoblasts was done as previously described.³⁷ Briefly, whole cell extracts or subcellular fractions were sonicated in RIPA buffer (20 mM Tris-HCl, pH 7.0, 1% nonidet P-40 (NP-40), 150 mM NaCl, 10% glycerol, 10 mM EDTA, 20 mM NaF, 5 mM sodium pyrophosphate, 1 mM Na₃VO₄, 1 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml pepstatin) at 4°C.

To obtain purified nuclear fractions, the cell pellet was resuspended in a lysis buffer containing 10 mM Tris, pH 7.8, 1% nonidet P-40 (NP-40), 10 mM 2-mercaptoethanol, and protease inhibitors. Separation of nuclei was obtained by hypotonic shock and shearing; nuclei were pelleted by 300×g centrifugation at 4°C.

Table 1 LMNA mutations reported in this study

Protein	CDNA	Gene location	Phenotype	Patients	References
R377H	1130G→T	Exon 6	LGMD 1B	Patient 1	Unpublished
R190Q and R249Q	569G→A 746G→A	Exon 4-exon 2	EDMD2	Patient 2	Unpublished
I63N	188T→A	Exon 1	EDMD2	Patient 3	Boriani <i>et al</i> , 2003 ³⁵
L140P	419T→C	Exon 2	EDMD2	Patient 4	Boriani <i>et al</i> , 2003 ³⁵
R527P	1580G→C	Exon 9	EDMD2	Patient 5	Bonne <i>et al</i> , 1999 ⁴

Figure 2 N-terminus lamin A phosphorylation is observed in cycling C2C12 myoblasts (cyc), it is strongly reduced in resting myoblasts (rest), while it is preserved in differentiating myotubes at days 2 (2d) or 4 (4d) of differentiation. N-terminus lamin C phosphorylation is modulated with a similar pattern, though a less intense phosphorylated lamin C band is observed in cycling myoblasts. The nitrocellulose membrane was re-probed with polyclonal antilamin A/C antibody (lower panel). Nuclear lysates were used for p-lamin A/C and lamin A/C western blot analysis. Whole cellular lysates from each fraction were subjected to SDS-PAGE and immunoblotted using troponin-T antibody as a marker of myogenic differentiation. Minor contamination by myotubes (troponin-T band) is detected in the resting myoblast fraction. p-Lamin A, phosphorylated lamin A; p-lamin C, phosphorylated lamin C.

Proteins were separated by SDS-PAGE, and immunoblotted with anti-lamin A/C (Santa Cruz Biotechnology, Santa Cruz, California, USA) or anti-SW2-30 antibody.³⁰

Immunofluorescence labelling

Cultured myoblasts grown on coverslips were fixed in methanol at -20°C . Unfixed cryosections of muscle samples and fixed cells were reacted with anti-lamin A/C (mouse monoclonal; Novocastra Laboratories, Newcastle upon Tyne, UK) or anti-SW2-30 diluted 1:100 and 1:30, respectively, in phosphate buffered saline containing 4% bovine serum albumin. Dephosphorylation of cryostat muscle sections with alkaline phosphatase (10 U/ml at 37°C for 30 minutes) was carried out to obtain a negative control of phosphorylated lamin A/C labelling. Antilaminin $\alpha 2$ chain staining (Chemicon) of cryostat muscle sections was done to distinguish muscle nuclei from interstitial cell nuclei, while anti-fetal-myosin heavy chain antibody (Novocastra) was employed to label regenerating fibres.

RESULTS

Lamin phosphorylation in living muscle cells

To evaluate the specificity of SW2-30 anti-lamin A/C antibody against the phosphorylated amino-terminus sequence of lamin A/C, we treated C2C12 mouse myoblast lysates with alkaline phosphatase and evaluated the presence of phosphorylated lamin A/C in the cellular lysate compared with untreated myoblasts. A doublet at 70 and 65 kDa was labelled by SW2-30 antibody in untreated myoblast lysates, while SW2-30 immunoreactivity was completely lost in phosphatase treated myoblasts (fig 1). It is worth noting that a hypophosphorylated lamin band was detected in phosphatase treated samples by polyclonal or monoclonal antilamin A/C antibodies just below the phosphorylated lamin band (fig 1). Although the precise epitope recognised by SW2-30 antibody has not been determined, this set of experiments allowed us to state that SW2-30 selectively labels phosphorylated lamin A/C in myoblasts.

Figure 3 N-terminus lamin A phosphorylation is increased by insulin treatment of cultured C2C12 myoblasts, while lamin C is not affected. Serum starved cells ($-FCS$) show a low level of lamin A/C phosphorylation. Insulin treatment ($+INS$) (10 μM) increased phosphorylated lamin A level (p-lamin A), but not phosphorylated lamin C (p-lamin C). p473Akt represents phosphorylated Akt band, here labelled to show activation of the insulin pathway. The immunoblotted membrane was re-probed with polyclonal antilamin A/C or actin to show equal loading amounts.

The presence of phosphorylated lamins was then evaluated in cycling as well as in quiescent C2C12 myoblasts (reserve cells) and in myotubes at day 4 in differentiation medium. A high degree of SW2-30 labelling was observed in cycling myoblasts and in myotubes by both immunofluorescence labelling of nuclei and western blot analysis of cellular lysates, while lamin phosphorylation was reduced in quiescent mononucleated cells (fig 2). We evaluated the possibility that insulin signalling could trigger lamin phosphorylation in myoblasts. Phosphorylation of lamin A/C by insulin has been reported previously in BHK-21 fibroblasts,²⁸ and we applied the same protocol to C2C12 myoblasts. Lamin A phosphorylation increased linearly in insulin treated cultures, depending on the dose employed (data not shown). Maximum phosphorylation was observed after treatment with 10 μM insulin (fig 3). On the other hand, lamin C phosphorylation was not affected by insulin treatment (fig 3). This finding indicates that activation of the insulin signalling pathway can lead to phosphorylation of lamin A in myoblasts.

Lamin phosphorylation in patient myoblasts and myotubes

The next step of our study was the evaluation of lamin A/C phosphorylation in cultured myoblasts from controls and from LGMD 1B and EDMD2 patients (table 1).

The differentiation rate of cultured EDMD2 myoblasts was determined and compared with control human myoblast cultures. A differentiation rate comparable to controls was observed in primary EDMD2 myoblasts at low passage number (two passages in culture medium) (data not shown). In control human myotubes, SW2-30 labelling was increased relative to undifferentiated cells (fig 4A), as previously observed in mouse myoblasts (fig 2). On the other hand, lamin A/C phosphorylation was reduced in EDMD2 myotubes relative to control human myotubes, and it was undetectable in EDMD2 reserve cells (fig 4A). The same reduction in lamin phosphorylation was observed in LGMD 1B myoblasts from patient 1 carrying the R377H lamin A/C mutation and in

Figure 4 Lamin A/C phosphorylation is reduced in EDMD2 and LGMD 1B myoblasts. Control human myotubes show bright fluorescence staining at the nuclear rim by SW2-30 antilamin A/C (p-lamin A/C). SW2-30 labelling is reduced in EDMD2 myotubes (EDMD2) and in LGMD 1B myotubes (LGMD 1B). (B) Western blot analysis shows that total lamin A/C level is slightly lowered in the EDMD2 case examined here (patient 2), but not in LGMD 1B cells. DAPI staining of nuclei is shown. These pictures are representative of results obtained using SW2-30 antibody in all reported cell lines.

EDMD2 myoblasts from patient 2 carrying an R190Q+R249Q mutation of lamin A/C (fig 4A). In myotubes from patient 2, the total amount of lamin A/C was slightly reduced, while expression of lamin A/C in myotubes from patient 1 was not affected (fig 4B).

Lamin phosphorylation in Emery–Dreifuss muscle fibres

Both the whole lamin A/C expression level and SW2-30 staining of nuclei were then evaluated in mature muscle fibres from either control or EDMD2 patient muscle biopsies (fig 5). A slightly reduced level of lamin A/C expression was observed in EDMD2 patient 2 bearing R190Q and R249Q *LMNA* mutations on the same allele (fig 5), while lamin A/C expression in the other EDMD2 muscles examined was comparable to controls (fig 5). Conversely, SW2-30 labelling was strikingly reduced in muscle fibres from all the EDMD2 and LGMD 1B cases examined (fig 5, and data not shown for the LGMD 1B patient), bearing different *LMNA* mutations (table 1).

Laminin staining of sarcoplasmic membrane allowed us to show that lamin phosphorylation is present in both muscle nuclei and non-muscle cells (fig 6A). In EDMD2 muscle biopsies, SW2-30 antibody labelling was completely absent from the nuclei of mature muscle fibres, while lamin A/C phosphorylation was still detected in nuclei from non-muscle

cells (fibroblasts and inflammatory cells; fig 6A, arrows). This finding suggests that a tissue specific mechanism, possibly involving selective targeting of a protein kinase to lamin A/C in muscle, might be altered in EDMD2.

Lamin phosphorylation in regenerating muscle fibres

SW2-30 lamin A/C labelling was strongly increased in regenerating muscle fibres (fig 6B), as determined in muscle biopsies from patients with various pathologies which show a high degree of fetal myosin heavy chain expression; a representative sample from a patient with Duchenne muscular dystrophy is shown in fig 6B. This result is in agreement with the observation that lamin phosphorylation is enhanced during myoblast activation (figs 2 and 4). It is noteworthy that SW2-30 staining was still detectable in regenerating EDMD2 muscle (fig 6B). Nevertheless, the fluorescence signal was reduced in regenerating EDMD2 muscle nuclei relative to regenerating muscle nuclei from pathological controls (fig 6B), suggesting a lower rate of lamin A N-terminal phosphorylation in EDMD2 muscle during the regeneration process.

DISCUSSION

This study is the first to report on lamin A/C phosphorylation in differentiating myoblasts and regenerating human skeletal muscle. Immunolabelling with a monoclonal antibody

Figure 5 Lamin A/C SW2-30 staining (P-lamin A/C) is reduced in all laminopathic muscle biopsies examined, while the total lamin A/C amount is only reduced in patient 2. See table 1 for patient details. A phosphatase treated control sample is shown in the lower row as a negative control for SW2-30 staining.

directed against the phosphorylated amino-terminus moiety of lamins A and C allowed us to show that lamin A/C phosphorylation at this site is increased in proliferating or differentiating myoblasts. Involvement of insulin signalling in lamin A N-terminus phosphorylation is demonstrated by insulin stimulation of serum starved myoblasts. This finding appears particularly relevant considering that insulin activation of the PI-3 kinase Akt pathway is a key event in myoblast proliferation and to a large extent in myoblast differentiation. It is noteworthy that two consensus sites for Akt have been determined around serine 301 and serine 404 in the lamin A/C sequence; thus it will be of interest to investigate the role of Akt in lamin A/C N-terminal phosphorylation.

Phosphorylation of lamin A/C by insulin was previously shown in BHK-21 cells.²⁸ The significance of lamin A/C phosphorylation in these cells is not obvious; however, the

investigators suggest that it might mediate chromatin reorganisation²⁸—a key event in muscle differentiation³⁸—which is affected in EDMD2 muscle.¹⁸ In the present study, we found that lamin A/C phosphorylation is strongly reduced in muscle cells bearing different *LMNA* mutations. As SW2-30 labelling was reduced in all the samples examined, altered lamin phosphorylation is likely to result from failure of lamin A/C to interact with a protein kinase that can bind lamin at multiple sites. An interesting aspect is that lamin A/C N-terminus phosphorylation is lost in laminopathic muscle but not in interstitial fibroblasts found in muscle biopsies. We speculate that different kinases might mediate lamin phosphorylation in fibroblasts and muscle cells. In this respect, it is worth noting that protein kinase C α has been shown to interact with lamin A in vitro,²⁹ and we confirmed this interaction in fibroblasts but not in cultured myoblasts (not shown). Therefore, EDMD2 mutations reported in the present study are likely to be irrelevant to protein kinase C α interaction with lamin A, while they could affect lamin A/C binding to a muscle specific kinase yet to be identified. Interestingly, it has recently been reported that mouse myoblast clones expressing mutated lamin A/C fail to differentiate properly.³⁹ Although we did not observe a reduced myoblast differentiation rate in the Emery–Dreifuss and limb girdle muscular dystrophy samples examined here, we cannot rule out the possibility that subsequent events in the differentiation process may be affected in laminopathic myoblasts, also as a result of impaired lamin phosphorylation.

The antibody employed in this study selectively labels a phospho-epitope located in the N-terminal moiety of lamins A and C.³⁰ We are now evaluating, by in vitro ³²P labelling of mutated lamin A/C, the possibility that phosphorylation of lamin A or C at other sites might be altered by pathogenic *LMNA* mutations.

The role of N-terminus phosphorylated lamin A/C in muscle remains to be elucidated. However, we suggest that lamin A phosphorylation may affect intermolecular interactions of the nuclear lamina constituent with either its partner proteins or chromatin. For instance, lamin A/C also interacts with actin at the nuclear level,^{37–40} and this interaction might be affected by the phosphorylation state of lamins. Along these lines, we recently showed that the interaction between emerin and actin in myoblasts is modulated through phosphorylation events.³⁷ Emerin is the nuclear envelope protein that is absent or mutated in EDMD1.⁴¹ Both emerin and lamin A/C interact with chromatin constituents^{3–42–43} as well as with nuclear actin,^{37–40} and a major role of such a protein complex in chromatin remodelling has been hypothesised.^{44–46} Interestingly, it has recently been reported that binding of lamin B receptor to chromatin is regulated by phosphorylation in the RS region.⁴⁷

We previously showed peripheral heterochromatin disorganisation in a proportion of nuclei from EDMD2 muscle.¹⁸ This defect could be related to reduced lamin A/C phosphorylation.²⁸ Lamin phosphorylation, however, appears to be reduced in all observed muscle nuclei. This discrepancy may reflect a dynamic situation, with lamin being mutated and unable to respond to signalling events, while secondary defects of chromatin arrangement appear in nuclei only under certain functional conditions or may selectively affect some chromatin districts.

The link between altered lamin A/C phosphorylation and defective intermolecular interaction of lamins with either their partner proteins or chromatin remains to be established. This report suggests two major lines of investigation: the physiological role of lamin A/C N-terminus phosphorylation in muscle cells, and the pathogenic mechanism involving altered lamin A/C phosphorylation in EDMD2 and LGMD 1B. In this respect, it should be noted that heterochromatin

Figure 6 (A) In control human muscle, SW2-30 (green) labels the nuclear rim from both muscle (arrowheads) and non-muscle cells (arrows), as determined by double staining with laminin $\alpha 2$ chain (red). In EDMD2 muscle only interstitial fibroblasts retain SW2-30 nuclear rim labelling (arrows). Nuclei are counterstained with DAPI. (B) In regenerating fibre nuclei, SW2-30 staining (green) is increased at the nuclear rim with respect to mature muscle nuclei. Muscle fibre nuclei are indicated by arrowheads and fibroblast nuclei by arrows. A muscle sample from Duchenne muscular dystrophy (DMD) has been labelled to evaluate regenerating fibres as a control. In EDMD2 and LGMD 1B muscle, regenerating fibres show reduced lamin SW2-30 staining with respect to regenerating fibres from DMD muscle, while fibroblast nuclei retain SW2-30 labelling comparable to control. A representative section from EDMD2 patient 4 muscle biopsy is shown here. Fetal myosin heavy chain labelling (red) was used as a marker of regenerating muscle fibres.

disorganisation and reduced lamin A/C N-terminus phosphorylation represent the only common defects so far identified in laminopathic muscle. This observation strongly suggests that both aspects are key events in EDMD2 and LGMD 1B pathogenesis and we hypothesise that lamin A/C N-terminal phosphorylation may be required for the inter-molecular interactions regulating proper heterochromatin organisation.⁴⁵

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