Specific phosphorylation of Ser458 of A-type lamins in *LMNA*-associated myopathy patients

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Summary

Mutations in *LMNA*, which encodes A-type nuclear lamins, cause various human diseases, including myopathy, cardiomyopathy, lipodystrophy and progeria syndrome. To date, little is known about how mutations in a single gene cause a wide variety of diseases. Here, by characterizing an antibody that specifically recognizes the phosphorylation of Ser458 of A-type lamins, we uncover findings that might contribute to our understanding of laminopathies. This antibody only reacts with nuclei in muscle biopsies from myopathy patients with mutations in the Ig-fold motif of A-type lamins. Ser458 phosphorylation is not seen in muscles from control patients or patients with any other neuromuscular diseases. In vitro analysis confirmed that only lamin A mutants associated with myopathy induce phosphorylation of Ser458, whereas lipodystrophy- or progeria-associated mutants do not. We also found that Akt1 directly phosphorylates Ser458 of lamin A with myopathy-related mutations in vitro. These results suggest that Ser458 phosphorylation of A-type lamins correlates with striated muscle laminopathies; this might be useful for the early diagnosis of *LMNA*-associated myopathies. We propose that disease-specific phosphorylation of A-type lamins by Akt1 contributes to myopathy caused by *LMNA* mutations.

Key words: Laminopathy, A-type lamins, EDMD, LGMD1B, Akt

Introduction

Mutations in *LMNA* cause at least 13 human hereditary diseases, collectively termed 'laminopathies'. These include striated muscle diseases, such as autosomal dominant and recessive forms of Emery-Dreifuss muscular dystrophy (AD/AR-EDMD) (Bonne et al., 1999; Raffaele Di Barletta et al., 2000), limb-girdle muscular dystrophy type 1B (LGMD1B) (Muchir et al., 2000), *LMNA*-related congenital muscular dystrophy (L-CMD) (Quijano-Roy et al., 2008) and dilated cardiomyopathy (Fatkin et al., 1999), and other conditions including Hutchinson-Gilford progeria syndrome (HGPS) (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003), atypical Werner syndrome (Chen et al., 2003), mandibuloacral dysplasia (MAD) (Novelli et al., 2002) and Dunnigan-type familial partial lipodystrophy (FPLD) (Shackleton et al., 2000). It is still not clear how mutations in *LMNA* cause such a wide variety of tissue-specific degenerative diseases, although A-type lamins are ubiquitously expressed.

A-type lamins, of which lamin A and lamin C are the predominant somatic cell isoforms, are type V intermediate filament proteins that form the nuclear lamina, a meshwork on the nucleoplasmic side of the inner nuclear membrane (Burke and Stewart, 2002; Capell and Collins, 2006). Like all intermediate filament proteins, lamins have a tripartite structure consisting of an N-terminal head, a central coiled-coil rod domain and a large globular C-terminal tail. The C-terminal tail domain has an immunoglobulin-like fold (Ig-fold) motif (residues 436–544); these motifs are known to be involved in protein–protein interactions (Dechat et al., 2000; Lee et al., 2001; Sakaki et al., 2001; Zastrow et al., 2006; Zastrow et al., 2004). In addition to their primary role in providing mechanical support for nuclear membranes to maintain nuclear shape and size (Goldman et al., 2004), lamin filaments are believed to play important roles in mitosis (Tsai et al., 2006), chromatin organization (Glass et al., 1993; Park et al., 2009; Taniura et al., 1995), transcription (Spann et al., 2002) and DNA replication (Moir et al., 2000; Spann et al., 1997).

According to the PhosphoSite database (http://www.phosphosite.org/), more than 30 phosphorylation sites in human A-type lamins have been reported. Phosphorylation of Thr19, Ser22 and Ser392 leads to depolymerization of lamin filaments during nuclear envelope breakdown in mitosis and meiosis (Haas and Jost, 1993; Heald and McKeon, 1990; Peter et al., 1990; Ward and Kirschner, 1990), but the physiological importance of the phosphorylation of other sites in A-type lamins is largely unknown. Previously, Cenni et al. reported that N-terminal phosphorylation of lamin A was specifically decreased in the muscles of four AD-EDMD or LGMD1B patients (Cenni et al., 2005), implicating unidentified lamin A phosphorylation sites in the pathomechanism of AD-EDMD and LGMD1B.

Here, we produced site- and phosphorylation-state-specific antibodies against human A-type lamins, and found that the antibody against phosphorylated Ser458 specifically detects myopathy patients who have mutations within the Ig-fold motif of the C-terminal tail domain of A-type lamins. In vitro expression analysis revealed that Ser458 phosphorylation was specific to myopathy-causing *LMNA* mutations, because it was not detected in cells with mutations related to FPLD or progeria. These results imply that Ser458 phosphorylation might have particular roles in the pathomechanism of *LMNA*-associated myopathy.

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Results

Ser458 phosphorylation of A-type lamins in the muscles of *LMNA*-associated myopathy patients

To examine the phosphorylation state of A-type lamins, we raised rabbit polyclonal antibodies against three phosphorylated A-type lamin peptides – Ser5-*P*, Thr416-*P* and Ser458-*P* (Fig. 1A); these serine and threonine residues are well conserved between species. We performed immunohistochemistry of muscle specimens from 17 genetically confirmed *LMNA*-associated myopathy patients, including AD-EDMD, LGMD1B and L-CMD patients.

Using the purified anti-phospho-Ser458 antibody (anti-Ser458-*P* Ab), we found clear nuclear staining in muscles from patients with *LMNA*-associated myopathies, but not in control muscles (Fig. 1Ba–d). Notably, strong immunoreaction to anti-Ser458-*P* Ab was observed in all eight patients carrying a mutation within the Ig-fold motif, whereas the remaining nine patients with mutations outside of the Ig-fold domain showed barely detectable nuclear staining (Table 1). Ser458 is an evolutionarily highly conserved residue within the Ig-fold motif (amino acids 436–544) of A-type lamins (supplementary material Fig. S1A). Anti-Ser458-*P* Ab staining was independent of patient age and sex, and independent of the nature or severity of myopathy, because positive staining for Ser458-*P* was seen in AD-EDMD, LGMD1B, L-CMD and infantile inflammatory myopathy (IIM) cases.

Positive staining with the anti-Ser458-*P* antibody was detected in myonuclei [both peripheral and centrally located (Fig. 1Bb,d,e,g)], vascular endothelial and smooth muscle cell nuclei (Fig. 1Bi), and the nuclei of unidentified cells outside the basal lamina (Fig. 1Bf,h). Double staining with anti-Ser458-*P* and anti-Pax7 antibodies also revealed positive staining in nuclei of satellite cells in patient muscles (Fig. 1Bj–l). Double staining with antibodies for Ser458-*P* and pan-A-type lamins showed that 69.0±7.3% of nuclei were immunostained in patient muscles with mutations in the Ig-fold domain. These results suggest that the Ser458 phosphorylation might be common in *LMNA*-associated myopathy patients with mutations in the Ig-fold domain of A-type lamins.

Consistent with the immunohistochemistry results, the anti-Ser458-*P* Ab detected two main bands at 70 kDa and 65 kDa, corresponding to lamins A and C, respectively, in muscle from laminopathy patients, but not in unaffected control muscle (Fig. 2).

No Ser458 phosphorylation of A-type lamins in patients with other neuromuscular disorders

To determine whether Ser458 phosphorylation is specific to *LMNA*-associated myopathy, we immunostained muscle specimens from patients with other neuromuscular diseases, including X-linked EDMD (X-EDMD (Fig. 3c,d), Duchenne muscular dystrophy (DMD) (Fig. 3e,f), Becker muscular dystrophy (BMD) (Fig. 3g,h), LGMD2A (Fig. 3i,j), LGMD2B (Fig. 3k,l), sporadic inclusion body myositis (sIBM) (Fig. 3,m,n), idiopathic polymyositis (PM) (Fig. 3o,p) and myotonic dystrophy type 1 (MyD1) (Fig. 3q,r). As shown in Fig. 3, Ser458 phosphorylation was only observed in

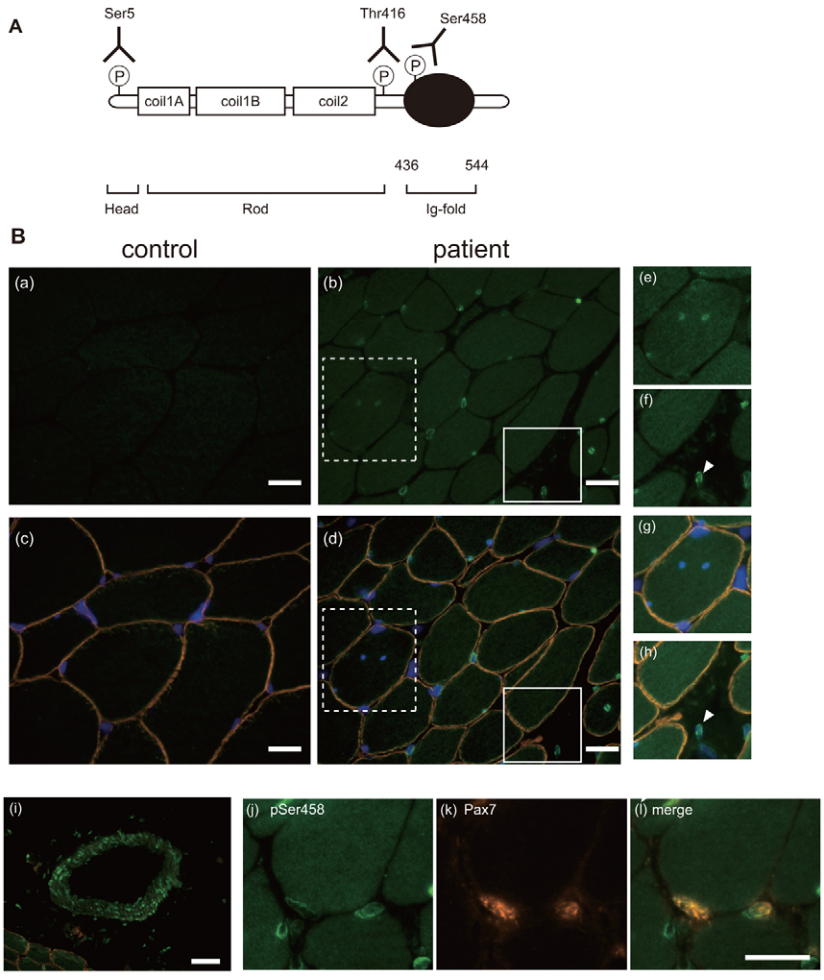

Fig. 1. Ser458 of A-type lamins is specifically phosphorylated in muscles from *LMNA*-associated myopathy patients. (**A**) Schematic model of three antibodies against phosphorylated A-type lamins (Ser5-*P* Ab, Thr416-*P* Ab and Ser458-*P* Ab). Amino acid residues 436–544 form an Ig-fold structure in the C-terminal globular tail domain. (**B**) Immunohistochemistry of human muscles with anti-Ser458-*P* antibody. Nuclei in patient muscle (P11) show positive staining for anti-Ser458-*P* antibody (b,d), whereas no positive nuclear staining is seen in control muscle (a,c). Anti-Ser458-*P* Ab is green and anti-merosin antibody is red. (c,d) Merged images. Blue is DAPI staining. (e,f) Magnified images of boxes in b. (g,h) Magnified images of boxes in d. Note that centrally placed nuclei and nuclei in non-muscle cells outside of the basal lamina (arrowheads) are immunostained with anti-Ser458-*P* Ab. (i) Nuclei in blood vessels in the patient muscle (P14) are also immunostained with anti-Ser458-*P* Ab. (j–l) Double staining of *LMNA*-associated myopathy patient muscle (P12) with anti-Ser458-*P* Ab (j) and anti-pax7 (k) reveals colocalization (l), indicating the occurrence of Ser458 phosphorylation in the patient satellite cells. Scale bars: 20 μm.

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Table 1. Summary of anti-Ser458-*P* antibody staining of A-type lamins from 17 *LMNA*-associated myopathy patients

Patient	Age	Sex	Clinical diagnosis	*LMNA* mutation	Domain	Anti-Ser458-*P* Ab
P1	3y 11m	F	LGMD1B	p.R28Q	Head	–
P2	2y 1m	F	IIM	p.K32 deletion	Head	–
P3	1y	M	IIM	p.R41S	Rod	–
P4	1y 6m	F	L-CMD	p.R41C	Rod	–
P5	2y 8m	F	LGMD1B	p.R249Q	Rod	–
P6	5y 3m	F	LGMD1B	p.S303P	Rod	–
P7	47y	M	LGMD1B	p.K311R	Rod	–
P8	2y 5m	M	IIM	p.E358K	Rod	–
P9	1y 9m	F	L-CMD	p.E444_D446 duplication	Tail (Ig fold)	+
P10	11y 1m	F	EDMD	p.R453W	Tail (Ig fold)	+
P11	4y 5m	F	LGMD1B	p.R453W	Tail (Ig fold)	+
P12	4y	F	LGMD1B	p.R453W	Tail (Ig fold)	+
P13	5y	F	IIM	p.N456H	Tail (Ig fold)	+
P14	7y 2m	M	LGMD1B	p.W514R	Tail (Ig fold)	+
P15	25y	F	LGMD1B	p.R527P	Tail (Ig fold)	+
P16	2y 11m	M	LGMD1B	p.T528K	Tail (Ig fold)	+
P17	52y	F	LGMD1B	p.V547X	Tail	–

patients with *LMNA*-associated myopathy (Fig. 3a,b), but not with other muscle diseases such as X-EDMD (Fig. 3c,d), which is clinically indistinguishable from AD-EDMD. This result suggests that Ser458 phosphorylation is likely to be specific to *LMNA* mutations.

In contrast to the anti-Ser458-*P* antibody, the anti-Ser5-*P* antibody strongly recognized nuclei in all human muscles examined, with no disease or mutation specificity. The anti-Thr416-*P* antibody showed very weak staining in nuclei, with no difference between control and patients (supplementary material Fig. S1B,C).

Ser458 phosphorylation of A-type lamins in AD-EDMD patient fibroblasts

Because Ser458 phosphorylation of A-type lamins was also observed in the nuclei of non-muscle tissues (Fig. 1B), we performed immunocytochemistry using cultured skin fibroblasts from one unaffected person and two AD-EDMD patients with either a Leu102Pro mutation in the rod domain or a Arg453Trp mutation in the Ig-fold domain. Anti-Ser458-*P* Ab staining was observed only in the fibroblasts with the Arg453Trp mutation, but not in control and Leu102Pro mutant fibroblasts (Fig. 4). All nuclei in the Arg453Trp fibroblasts were immunopositive, implying that Ser458 phosphorylation might be independent of the cell cycle. These results suggested that Ser458 phosphorylation requires mutations in the Ig-fold motif of A-type lamins and can occur in non-muscle cells.

Ser458 phosphorylation of A-type lamins in transfected cells

Next, we analyzed the specificity of anti-Ser458-*P* Ab to phosphorylation by examining ectopically expressed FLAG-tagged lamin A and FLAG-tagged Arg453Trp mutant lamin A, which is the most common mutation in *LMNA*-associated myopathy. Anti-Ser458-*P* Ab strongly detected the ectopic FLAG–Arg453Trp mutant in a western blot analysis and slightly detected even wild-type lamin A when overexpressed in COS-7 cells (Fig. 5A). Importantly, alkaline phosphatase treatment reduced the immunoreactivity of anti-Ser458-*P* Ab to background levels (Fig. 5A, IP), strongly suggesting that anti-Ser458-*P* Ab specifically recognizes the phosphorylation of Ser458 of mutant A-type lamins.

To further verify the specificity of anti-Ser458-*P* Ab, we transfected C2 myoblasts to express FLAG-tagged wild-type lamin A or FLAG-tagged lamin A bearing either the Ser458Ala or Arg453Trp mutation, or both mutations (FLAG–Arg453Trp/Ser458Ala) (Fig. 5B). Anti-Ser458-*P* Ab immunostained only the nuclei that expressed FLAG–Arg453Trp, whereas the nuclei that expressed FLAG–Arg453Trp/Ser458Ala were barely detectable with anti-Ser458-*P* Ab. These results confirmed the specificity of anti-Ser458-*P* Ab to Ser458 phosphorylation.

Ser458 phosphorylation is not detected in other laminopathies

As several mutations associated with FPLD, MAD and HGPS are known to be located within the Ig-fold motif of A-type lamins, we wanted to find out whether Ser458 phosphorylation also occurs in such mutants. We therefore transfected lamin A mutants associated with AD-EDMD (Leu140Pro, Arg453Trp, Arg527Pro, Leu530Pro) (Bonne et al., 1999; Boriani et al., 2003), LGMD1B (Tyr481His) (Kitaguchi et al., 2001), FPLD (Gly465Asp, Arg482Trp, Lys486Asn) (Shackleton et al., 2000; Speckman et al., 2000), MAD (Arg471Cys and Arg527His) (Cao and Hegele, 2003; Novelli et al., 2002) and HGPS (Met540Thr) (Verstraeten et al., 2006) into C2 myoblasts, and checked immunoreactivity with anti-Ser458-*P* Ab. As expected, Ig-fold mutants Arg453Trp, Arg527Pro, Leu530Pro and Tyr481His were detected by anti-Ser458-*P* antibody (Fig. 6). By contrast, rod domain mutant Leu140Pro formed intranuclear foci that were not detected by anti-Ser458-*P* Ab. Interestingly, lamin A mutations associated with FPLD (Gly465Asp, Arg482Trp, Lys486Asn), MAD (Arg471Cys, Arg527His) and

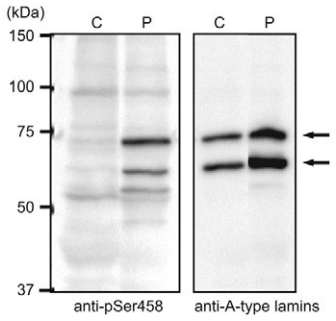

Fig. 2. Western blot analysis of patient muscle with anti-Ser458-*P* Ab. Anti-Ser458-*P* Ab specifically recognizes proteins of ~70 kDa and 65 kDa, corresponding to lamin A and lamin C, respectively. Representative data from patient 13 (P13) are shown. C: control, P: patient.

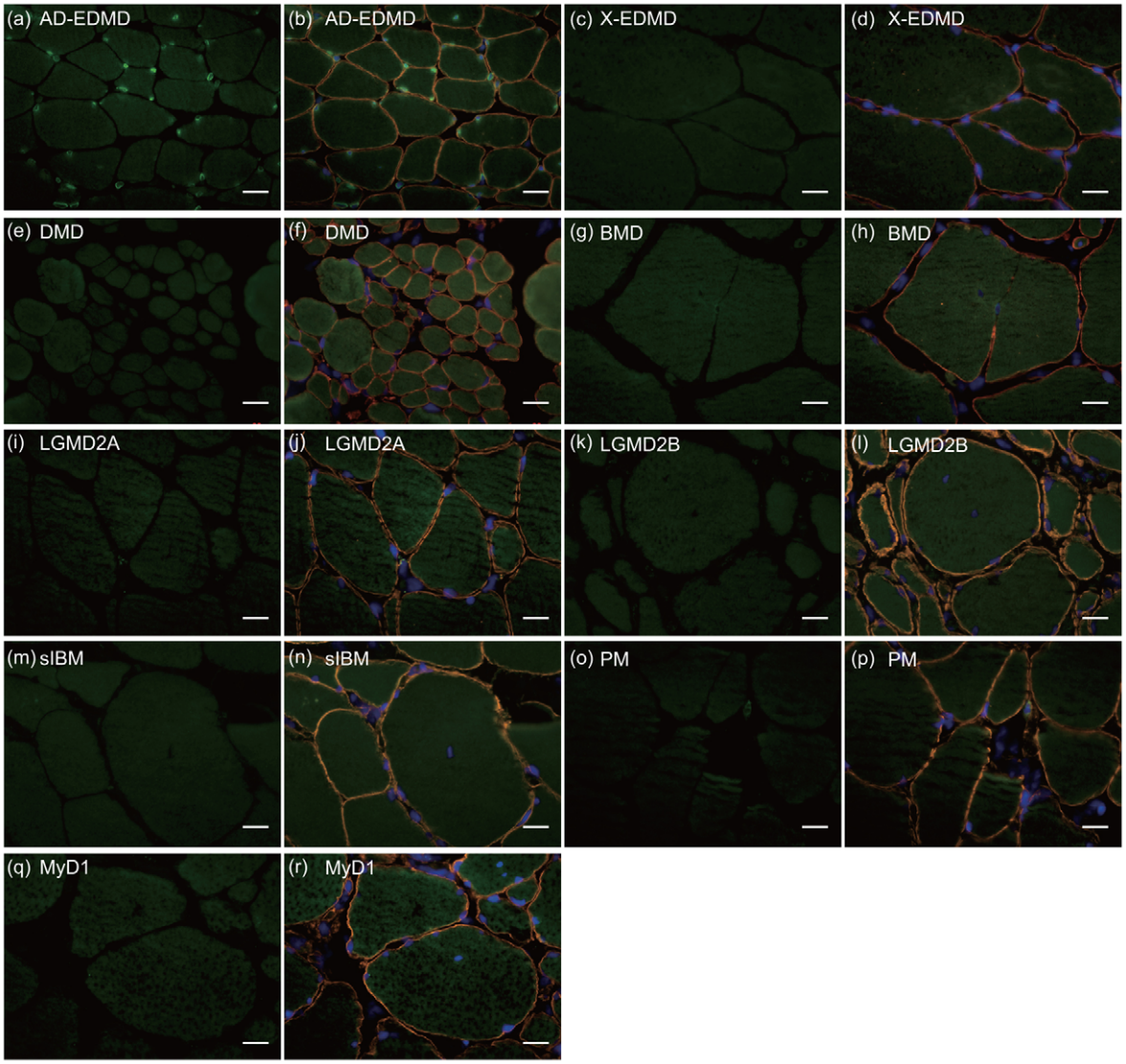

Fig. 3. Immunohistochemical analysis of other neuromuscular diseases. Muscles from myopathic patients were immunostained with anti-Ser458-*P* Ab (green) and anti-merosin (red). (**a**,**b**) AD-EDMD with Arg453Trp mutation in *LMNA* (P11), (**c**,**d**) X-EDMD, (**e**,**f**) DMD, (**g**,**h**) BMD, (**i**,**j**) LGMD2A, (**k**,**l**) LGMD2B, (**m**,**n**) sIBM, (**o**,**p**) PM, (**q**,**r**) MyD1. (b,d,f,h,j,l,n,p,r) Merged images. Nuclei were stained with DAPI (blue). Scale bars: 20 µm.

HGPS (Met540Thr), which are all located in the Ig-fold motif, were negative for anti-Ser458-*P* Ab. These results suggested that Ser458 in A-type lamins might be specifically phosphorylated in laminopathies related to myopathy, but not in other laminopathies even with mutations in the Ig-fold domain.

Akt1 directly phosphorylates Ser458 of lamin A

The sequence around Ser458 contains the Akt consensus sequence RxRxxS. To test whether Akt phosphorylates Ser458 of lamin A, COS-7 cells were co-transfected with wild-type Akt (wtAkt–HA) or myristoylated Akt (myrAkt–HA), which is regarded as a constitutively active form (Andjelkovic et al., 1997; Manning and Cantley, 2007), and FLAG-tagged lamin A constructs. The cell lysates were immunoprecipitated with FLAG M2 agarose and detected with anti-Ser458-*P* Ab. Coexpression of wtAkt–HA enhanced Ser458 phosphorylation of the Arg453Trp mutant; this signal became even stronger in cells co-transfected with myrAkt–HA (Fig. 7A). By contrast, only background levels of Ser458 phosphorylation were detected for wild-type lamin A and the double (Arg453Trp/Ser458Ala) mutant (Fig. 7A). The same results were obtained by co-transfection in C2 myotubes (supplementary material Fig. S2). To determine whether Ser458 phosphorylation is sensitive to the specific amino acid present at position Arg527, we co-transfected COS-7 cells with wtAkt–HA plus either the myopathy-causing Arg527Pro mutation or the MAD-causing Arg527His mutation of FLAG-tagged lamin A (Fig. 7B). Only background levels of Ser458 phosphorylation were detected with wild-type and His-substituted lamin A, suggesting specific recognition of myopathic Arg527Pro-mutated lamin A by Akt1.

To determine whether Akt1 directly phosphorylates Ser458 of lamin A, we performed an in vitro kinase assay using the purified C-terminal tail domain of lamin A (LA-T, amino acids 411–553) and a recombinant, constitutively active form of Akt1 (rAkt).

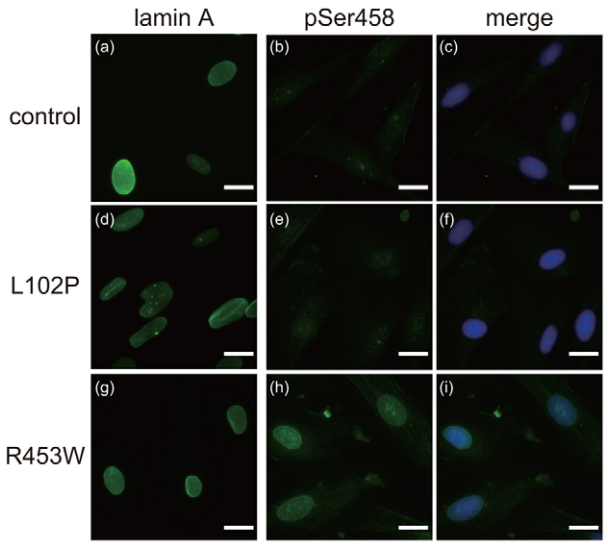

Fig. 4. Immunocytochemistry of human fibroblasts. Only fibroblasts from an AD-EDMD patient with an Arg453Trp mutation in the Ig-fold domain are detected with anti-Ser458-*P* Ab (h), whereas nuclear staining of cells from a control and an AD-EDMD patient with a Leu102Pro mutation is barely detectable (b,e). (**a**,**d**,**g**) Staining with anti-lamin A antibody, (**b**,**e**,**h**) staining with anti-Ser458-*P* Ab, (**c**,**f**,**i**) merged images of anti-Ser458-*P* Ab and DAPI. Scale bars: 20 μm.

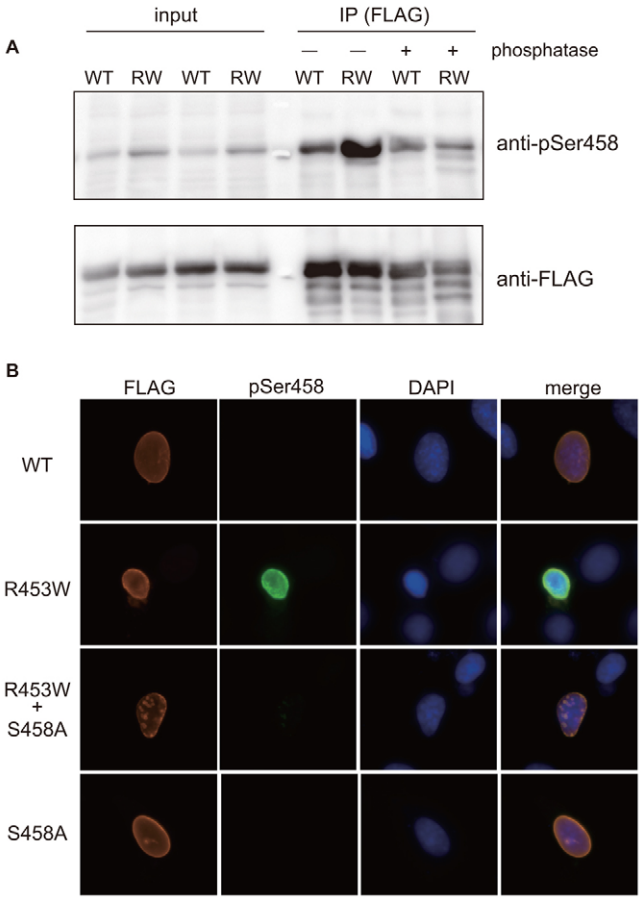

Fig. 5. Anti-Ser458-*P* Ab specifically recognizes the phosphorylation of Ser458 of lamin A. (**A**) Western blot analysis of transfected cells. FLAG-tagged lamin A constructs were overexpressed in COS-7 cells. The cell lysates were subjected to immunoprecipitation with anti-FLAG M2 antibody. The immunoprecipitates were incubated with (+) or without (–) calf intestine alkaline phosphatase, and then resolved by SDS-PAGE. WT: wild type, RW: Arg453Trp mutant. (**B**) Substitution of Ser458 to Ala diminishes the immunoreactivity of anti-Ser458-*P* against Arg453Trp mutant lamin A. Wild-type or mutant lamin A were overexpressed in C2 myoblasts, and then transfected cells were double immunostained with anti-FLAG Ab (red) and anti-Ser458-*P* Ab (green). Nuclei were stained with DAPI (blue).

Consistently, LA-T with the Arg453Trp mutation was phosphorylated by rAkt in vitro (Fig. 7C). rAkt failed to phosphorylate LA-T with Arg453Trp/Ser458Ala double mutations, indicating that Ser458 is a genuine Akt phosphorylation site. We conclude that Ser458 in lamin A is specifically phosphorylated by Akt1, even in non-muscle cells, when the Ig-fold domain bears mutations that cause myopathy; Ser458 is not phosphorylated when lamin A bears mutations, even in the Ig-fold, that cause disease in other tissues.

Discussion

For the first time, we have identified a disease-related phosphorylation site of A-type lamins using the antibody that specifically recognizes the phosphorylation of Ser458 of A-type lamins. Cenni et al. previously reported a monoclonal antibody, named SW2-30, that recognizes the phosphorylation of the N-terminal region of A-type lamins in normal muscles, but immunoreactions were barely detectable in myonuclei from AD-EDMD and LGMD1B patients (Cenni et al., 2005). The precise phosphorylation site was not determined, but the change in phosphorylation state between control and patients was the reverse of that seen with Ser458. In contrast to previously reported cell-cycle-specific phosphorylation (Heald and McKeon, 1990; Peter et al., 1990; Ward and Kirschner, 1990), Ser458 phosphorylation is independent of cell cycle, because the all nuclei in replicating fibroblasts from patients with the Arg453Trp mutation were immunopositive.

The early diagnosis of *LMNA*-associated myopathy is particularly important because the patients eventually develop severe cardiac problems with conduction defects, with high mortality (Bonne et al., 2003; Taylor et al., 2003). Because patients show a wide variety of clinicopathological features and recent research has revealed the spectrum of *LMNA*-associated myopathy to be broader than previously understood (Quijano-Roy et al., 2008), simple screening using disease-specific phospho-A-type lamin antibodies should be quite useful. Interestingly, the position of the mutation in *LMNA* is important for positive staining by anti-Ser458-*P* Ab (Table 1). About 30% of AD-EDMD and LGMD1B patients previously reported have the mutation within the Ig-fold motif (Leiden Muscular Dystrophy Pages; http://www.dmd.nl/). The Arg453Trp mutation, which is the most common mutation in AD-EDMD, is also located within this motif. Anti-Ser458-*P* antibody also detected the nuclei in skin fibroblasts and the vascular endothelial cells of patients, suggesting that skin biopsy, which is less invasive for the patient, might be used for the diagnosis.

We have revealed that Akt1 phosphorylates Ser458 of myopathy-related mutant lamin A. A recent study also reported that Ser404 of lamin A is phosphorylated by Akt (Cenni et al., 2008). Interestingly, Ser458 of wild-type lamin A was not phosphorylated by Akt1 even in vitro (Fig. 7C), suggesting that

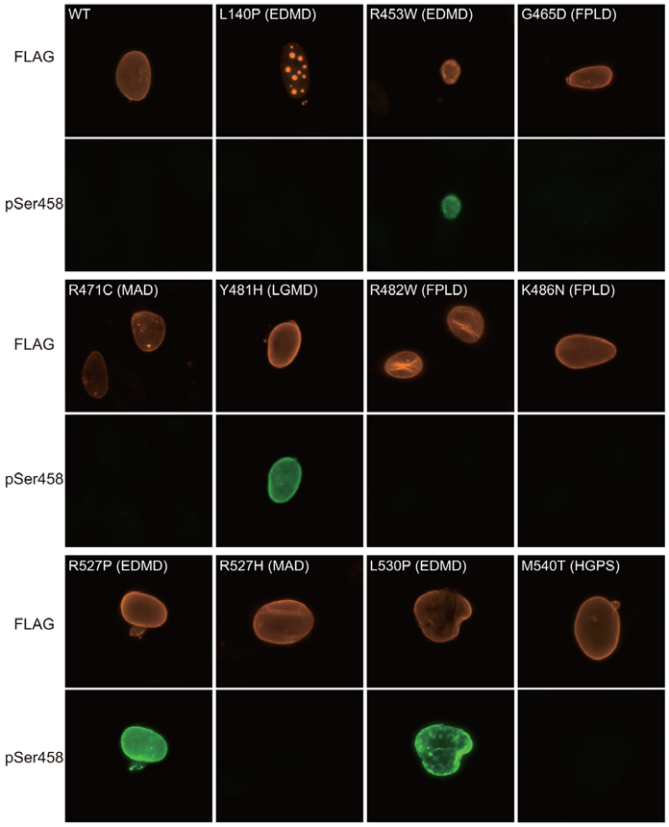

Fig. 6. Myopathy-specific phosphorylation of Ser458 of lamin A. Lamin A mutants that cause AD-EDMD, LGMD1B, FPLD, HGPS or MAD were overexpressed in C2 myoblasts. Transfected cells were double immunostained with anti-FLAG Ab (upper panel, red) and anti-Ser458-*P* Ab (lower panel, green).

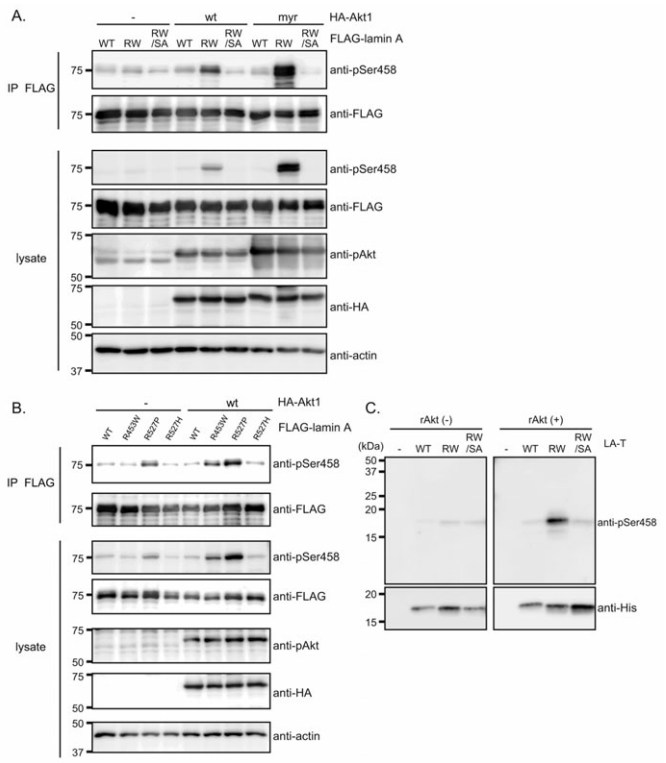

Fig. 7. Akt1 directly phosphorylates Ser458 of lamin A with myopathy-related mutations. (**A**) COS-7 cells were transfected with Akt–HA constructs (wtAkt–HA or myrAkt–HA) and FLAG–lamin A constructs (WT: wild type, RW: Arg453Trp mutant, RW/SA: Arg453Trp/Ser458Ala mutant) for 30 hours. FLAG–lamin A was immunoprecipitated (IP), then probed with anti-Ser458-*P* and anti-FLAG M2 antibodies. Whole-cell lysates were also probed with anti-phospho-Akt Ser473 (anti-pAkt), and anti-HA and anti-actin antibodies to confirm equivalent levels of expression. (**B**) COS-7 cells were transfected with FLAG–lamin A constructs alone or co-transfected with wtAkt–HA for 30 hours. FLAG–lamin A was immunoprecipitated, then immunoblotted as described above. (**C**) Purified His-tagged C-terminal tail domain of lamin A (LA-T) was phosphorylated in vitro in the presence (+) or absence (–) of recombinant active Akt (rAkt). The membranes were probed with anti-Ser458-*P* and anti-His tag antibodies.

Ser458 is buried in wild-type lamin A. Ser458 was found to be partly buried and structural studies have showed that the Arg453Trp mutation destabilizes the three-dimensional structure of the Ig-fold motif, whereas the mutations observed in FPLD (Arg482Trp and Arg482Gln) do not (Krimm et al., 2002). We propose that *LMNA* mutations that make Ser458 easily accessible to Akt1 phosphorylation predispose patients to muscle pathology.

It is nonetheless perplexing that EDMD and LGMD1B patients with mutations outside the Ig-fold domain have no detectable Ser458 phosphorylation. We speculate that mutations outside the Ig-fold motif might cause myopathy by different mechanisms, for example, by disrupting protein–protein interactions or lamin filament formation, because rod domain mutations such as Leu140Pro can disrupt polymerization and mislocalize lamins. Although further studies are needed to understand these mechanisms, antibodies that recognize Ser458-phosphorylated lamin A will be immediately useful for the differential diagnosis of a large fraction of *LMNA*-associated myopathies.

Materials and Methods

Clinical materials

All clinical materials used in this study were obtained for diagnostic purposes with informed consent. The studies were approved by the Ethical Committee of the National Center of Neurology and Psychiatry.

Mutation analysis

Genomic DNA was isolated from peripheral lymphocytes or muscle specimens using standard techniques. All *LMNA* exons and their flanking intronic regions were sequenced directly for all 17 patients using an ABI PRISM 3100 automated sequencer (PE Applied Biosystems, Foster City, CA). Primer sequences are shown in supplementary material Table S1.

Antibody production and purification

The following peptides for immunization were synthesized by MBL (Ina, Nagano, Japan). For phospho-A-type lamins: Ser5-*P* (Met-Glu-Thr-Pro-phosphoSer-Gln-Arg-Arg-Ala-Thr-Cys); Thr416-*P* (AcetylVal-phosphoThr-Lys-Lys-Arg-Lys-Leu-Glu-Cys); Ser458-*P* (Cys-Leu-Arg-Asn-Lys-phosphoSer-Asn-Glu-Asp-Gln-Ser). For non-phospho-A-type lamins: Ser5 (Met-Glu-Thr-Pro-Ser-Gln-Arg-Arg-Ala-Thr-Cys); Thr416 (AcetylVal-Thr-Lys-Lys-Arg-Lys-Leu-Glu-Cys); Ser458 (Cys-Leu-Arg-Asn-Lys-Ser-Asn-Glu-Asp-Gln-Ser).

To obtain the specific antibody for phospho-A-type lamins, rabbits were immunized with the phospho-peptides conjugated to keyhole limpet hemocyanin (KLH). Antisera were purified by affinity chromatography at 4°C. First, antisera were passed through HiTrap NHS-activated HP columns (GE Healthcare UK, Buckinghamshire, UK) coupled with non-phospho-A-type lamin peptides. Then, the flow-through fractions were collected. Next, the flow-through fractions were passed through the columns coupled with phospho-A-type lamin peptides. Antibodies were eluted with 0.15 M glycine–HCl (pH 2.7), concentrated by Amicon ultra-4 (Millipore, Bedford, MA, USA) and dialyzed with PBS at 4°C overnight. Approximately 0.2 μg/μl of antibody was obtained.

Immunohistochemistry

Biopsied muscle specimens were frozen in isopentane cooled in liquid nitrogen. Serial frozen sections of 6 μm thickness were fixed in cold acetone for 5 minutes at

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room temperature. After blocking with PBS containing 2% BSA and 5% heat-inactivated normal goat serum, the sections were incubated with primary antibodies for 2 hours at 37°C. Primary antibodies used in this study are: rabbit anti-phospho-A-type lamin polyclonal antibodies at 1:50; mouse anti-human merosin (M-chain) monoclonal antibody (5H2) (Chemicon International, Temecula, CA) at 1:400; rabbit anti-lamin A polyclonal antibody (Sakaki et al., 2001) at 1:400; mouse anti-Pax7 monoclonal antibody (Developmental Studies Hybridoma Bank, Iowa City, IA) at 1:300. Sections were incubated with anti-rabbit IgG antibody conjugated with Alexa-Fluor-488 and anti-mouse IgG antibody conjugated with Alexa-Fluor-568 (Invitrogen, Carlsbad, CA) at 1:500 for 45 minutes at room temperature. To enhance the immunoreactions, we used Can Get Signal immunostain solution A (Toyobo, Osaka, Japan) to dilute primary and secondary antibodies. Sections were observed under a Zeiss Axiophot 2 microscope (Carl Zeiss, Oberkochen, Germany).

Immunoblot analysis

For biopsied muscle specimens, 20 slices of 10 μm cryosections were immediately sonicated in SDS sample buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 1% glycerol (v/v), 0.1% bromophenol blue, 6% 2-mercaptoethanol] and boiled for 5 minutes at 95°C. The lysates were centrifuged at 100,000 ***g*** at 4°C for 5 minutes. The supernatants were subjected to SDS-PAGE and the proteins were transferred to Immobilon-P membranes (Millipore). The membranes were blocked with blocking buffer [2% BSA in Tris-buffered saline (TBS) containing 0.05% Tween-20] at room temperature for 1 hour, then incubated with anti-Ser458-*P* antibody diluted in Can Get Signal solution 1 (Toyobo) at 1:1000 at 4°C overnight. The anti-Ser458-*P* antibody was followed with Histofine simple stain MAX-PO (Nichirei Biosciences, Tsukiji, Tokyo, Japan) diluted in Can Get Signal solution 2 at 1:500 at room temperature for 45 minutes. Recognized proteins were visualized by enhanced chemiluminescence plus detection reagent (GE Healthcare).

Plasmid construction and mutagenesis

To generate FLAG-tagged wild-type human lamin A, the N terminus of human lamin A was amplified by PCR using the following primers: 5′-GGAATTCA-CCATGGACTACAAAGACGATGACGACAAGGAGACCCCGTCCCAGCGG-3′ and 5′-CTCGCGGCTGACCACCTCTT-3′. PCR product was digested with *Eco*RI and *Acc*I, and subcloned into full-length human prelamin A in pUC19 (a kind gift from Howard J. Worman, Columbia University, NY). FLAG-tagged lamin A cDNA was cut out by digestion with *Eco*RI and *Xba*I, and subcloned into pcDNA3.1 (Invitrogen). Each of the mutant lamin A constructs was made by site-directed mutagenesis (Horton et al., 1989) using the primers listed in supplementary material Table S2. Arg453Trp and Arg471Cys mutants were made by combining primers LMNA1386 and R453W-Fw with R453W-Rv and LMNA2579, and LMNA1386 and R471C-Fw with R471C-Rv and LMNA2579, respectively.

Human full-length Akt1 was amplified by PCR using an Akt1 cDNA construct (a gift from Yukiko Gotoh, University of Tokyo, Japan) (Masuyama et al., 2001) and subcloned into pcDNA3 (Invitrogen). Then, a hemagglutinin (HA) tag was added at the C-terminal end, yielding wtAkt–HA. The constitutively active form of Akt (myrAkt–HA) was generated by adding a myristoylation site derived from murine Src tyrosine kinase to the N-terminal end of the Akt–HA described above (Andjelkovic et al., 1997; Manning and Cantley, 2007).

For the in vitro kinase assay, the C-terminal tail domain of lamin A (amino acids 411–553) was amplified by PCR using the following primers: 5′-CATATGG-GTGGGGGCAGCGTCACCAAAAAG-3′ and 5′-GGGATCCTTAGTCGTCCT-CAACCACAGTCACTGAGC-3′. PCR product was ligated with pGEM-T-easy (Promega, Madison, WI), digested with *NdeI* and *BamHI*, and subcloned into pET 15b vector (Merck, Darmstadt, Germany).

The sequences of all constructs were verified by DNA sequencing.

Cell culture and transfection

African green-monkey kidney fibroblast cell line COS-7 cells and mouse myoblast cell line C2 cells were cultured in DMEM (Sigma, St Louis, MO) supplemented with 10% FBS (Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2. Before transfection, the medium was replaced with serum-free DMEM. The cells were transiently transfected using FuGENE HD transfection reagent (Roche Diagnostics, Indianapolis, IN). After 8–12 hours transfection, DMEM supplemented with 20% FBS was added to the dishes to adjust the FBS concentration in the medium to 10%. The cells were used for each experiment 48 hours after transfection.

Immunoprecipitation and alkaline phosphatase treatment

COS-7 cells were transfected with 10 μg wild-type lamin A or Arg453Trp lamin A construct. The cells were lysed in 1.0 ml lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate and Complete Protease Inhibitor Cocktail (Roche Diagnostics). The lysates were incubated at 4°C for 30 minutes with gentle rotation and then centrifuged at 15,000 ***g*** at 4°C for 30 minutes. The supernatants were collected and their protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA). For immunoprecipitation, the protein concentration of the cleared lysates was adjusted to 1.0 μg/μl and 40 μl anti-FLAG M2 affinity gel (Sigma) was added. The mixtures were incubated at 4°C overnight. The resulting immune complexes were washed three times with TBS and two times with alkaline phosphatase buffer (AP buffer) containing 50 mM Tris-HCl (pH 9.0) and 1 mM $MgCl_2$. The immune complexes were incubated with calf intestine alkaline phosphatase (Takara Bio, Shiga, Japan) in AP buffer at 37°C overnight. The proteins were eluted by boiling at 95°C for 5 minutes in SDS sample buffer. Immunoblot analysis was performed as described above. To detect FLAG-tagged lamin A, we used mouse anti-FLAG M2 monoclonal antibody (Sigma) diluted in PBS containing 5% skim milk at 1:2500.

Immunocytochemistry

Human skin fibroblasts from healthy individuals (control), AD-EDMD patients with a *LMNA* Leu102Pro or Arg453Trp mutation, and transfected C2 myoblasts were plated onto 12 mm cover glass coated with type I collagen. The cells were fixed in methanol for 10 minutes at –20°C. The steps were as described under Immunohistochemistry.

Akt kinase assay

In mammalian cell experiments, COS-7 cells or C2 myoblasts were co-transfected with 5 μg each of Akt–HA construct and FLAG–lamin A construct. The cells were lysed in 1.0 ml lysis buffer supplemented with 50 mM sodium fluoride, 1 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride. FLAG–lamin A was immunoprecipitated as described above. The resulting immune complexes were washed two times with lysis buffer and two times with TBS. The proteins were eluted by boiling at 95°C for 5 minutes in SDS sample buffer. Immunoblot analysis was performed as described above. For the detection of phosphorylated proteins, Blocking One-P blocking solution (Nacalai Tesque, Kyoto, Japan) was used. Mouse anti-HA monoclonal antibody (16B12 clone; Covance, Princeton, NJ) was used at 1:1000, rabbit anti-phospho-Akt (Ser473) monoclonal antibody (Cell Signaling Technology, Danvers, MA) was used at 1:1000, and rabbit anti-actin polyclonal antibody (Nichirei) was used at 1:2000.

For the in vitro kinase assay, the C-terminal tail domain of lamin A (LA-T) was in-vitro translated as a His-tagged protein using the S30 T7 High-Yield Protein Expression System (Promega) according to the manufacturer's instructions. The LA-T was purified with Ni-NTA agarose and eluted with elution buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, followed by dialysis against 20 mM MOPS-NaOH (pH 8.0), 1 mM EDTA, 5% glycerol, 50 mM NaCl at 4°C overnight. Approximately 0.5 μg His-tagged protein was incubated with 5 ng recombinant active Akt1 (Millipore) in a reaction buffer consisting of 8 mM MOPS-NaOH (pH 7.0), 0.2 mM EDTA, 10 mM magnesium acetate, 100 mM ATP at 30°C for 30 minutes. The reaction was terminated by the addition of SDS sample buffer. Immunoblot analysis was performed as described above. For the detection of His-tagged proteins, mouse anti-His-tag monoclonal antibody (Merck) was used at 1:2000.

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