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Phosphorylation of a proline-directed kinase motif is responsible for structural changes in myogenin

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Abstract Myogenin, a member of the MyoD family which governs skeletal muscle differentiation, was identified as a pair of phosphorylated bands on SDS-PAGE during myogenesis. The slow migrating form was found to be hyperphosphorylated myogenin. In vitro phosphorylation by CDC2 kinase caused a prominent reduction in electrophoretic mobility of myogenin. Furthermore, we demonstrated that phosphorylation of the serine residue at position 43 contributes to the modification of myogenin in vivo and in vitro resulting in the reduction in electrophoretic mobility. We propose here that a CDC2-like proline-directed kinase regulates myogenin activity through its phosphorylation.

Key words: Myogenin; Myogenesis; CDC2 kinase; Cyclin-dependent kinase; Phosphorylation

1. Introduction

Myogenic determining factors including MyoD1, myogenin, Myf5, and MRF4, play a crucial role in the determination and terminal differentiation of myogenic cell lineage [1–7]. Gene disruption experiments have demonstrated that myogenin function is essential for myogenic differentiation and cannot be compensated for by other MyoD family members [8,9]. Thus, the regulation of myogenin function appears to be crucial for the terminal differentiation of myogenic cells. Myogenin activity is regulated at the post-translational level as well as the transcriptional level as shown for other myogenic determining factors (reviewed in [10]).

We have identified differentiating myoblasts as myogenin-positive mononucleated cells (Hashimoto et al., submitted). Terminal differentiation of differentiating myoblasts is canceled by growth factors, oncogene products, and chemical reagents modifying the phosphorylation state of cellular proteins ([11–19], Hashimoto et al., submitted). Thus, the decision between proliferation and differentiation would be made in differentiating myoblasts. It is conceivable that post-translational modification of myogenin is important for its activity and for switching from proliferation to terminal differentiation. In fact, calcium-dependent protein kinase (PKC) [20] and cAMP-dependent protein kinase (PKA) [21] phosphorylate overexpressed myogenin in non-myogenic cells (COS-1). However, it is unclear whether the phosphorylation of myogenin by PKC or PKA plays a role in myogenic differentiation. During myogenesis, myogenin is detected as a pair of bands on SDS-PAGE ([22]; Hashimoto et al., submitted) of which the slow migrating band is assumed to be a phosphorylated form. However, the phosphorylation state of myogenin in differentiating myoblasts and myotubes has not been described. Here we identify myogenin in myogenic cells as a pair of differentially phosphorylated forms with different electrophoretic mobilities. The slow migrating band is a hyperphosphorylated form whose mobility-shift implies a significant structural change in the myogenin protein, that is expected to affect its function. We found that CDC2 kinase, initially reported as a cytoplasmic cell cycle regulator in various animals [23–27], shifts the electrophoretic mobility of myogenin through phosphorylation in vitro. Furthermore, we determine the phosphorylation site responsible for the electrophoretic mobility-shift of myogenin by site-directed mutagenesis. This phosphorylation of myogenin may be important in the post-translational regulation of myogenin function during myogenesis.

2. Materials and methods

2.1. Cell culture and transfection

C2C12, 10T1/2, and COS-7 cells were maintained in growth medium consisting of Dulbecco's modified Eagle's minimum essential medium with 4.5 g/l glucose (DMEM) supplemented with 10% fetal calf serum (FCS; HyClone) under a humidified atmosphere containing 10% CO_2. For the induction of myogenic differentiation, the cells were cultured in DMEM plus 2% horse serum (HS/DMEM). 10T1/2 cells were transfected by calcium phosphate precipitation [28]. Wild type or mutant rat myogenin cDNA [7] was subcloned into the *Eco*RI site of an expression vector, pcDLSRα296 [29], and cotransfected to 10T1/2 cells with pSV2brs ([30]; Funakoshi Co., Tokyo). Myogenin-expressing cell clones were selected and maintained in DMEM supplemented with 10% FCS and blasticidin *S*-hydrochloride (4 μg/ml; Funakoshi Co., Tokyo). COS-7 cells were transiently transfected with myogenin expression plasmids using a transfection reagent, DOTAP (Boeringer Mannheim Biochemica). Cells were harvested 3 days later for immunoblotting as described (Hashimoto et al., submitted).

2.2. In vivo labeling and immunoprecipitation

C2C12 cells were cultured in growth medium to 70% confluence at which time the medium was switched to HS/DMEM. Four days later myotube cultures were labeled with 0.25 mCi/ml [^{32}P]orthophosphate (ICN Radiochemicals) for 4 h. Dishes were then rinsed 3 times with HEPES-buffered saline (10 mM HEPES-NaOH, pH7.4, 150 mM NaCl). After adding 200 μl of RIPA buffer (10 mM HEPES-NaOH, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 mM EDTA, 2.5 mM EGTA, 10 mM NaF, 5 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate), the cells were scraped and sonicated. Following 10 min centrifugation, the supernatant was frozen in liquid nitrogen and stored at −80°C until use. An aliquot of supernatant (120 μl) was precleared with 20 μl of 10% formalin-fixed *Staphylococcus aureus* (SA) conjugated with normal rabbit IgG. The precleared

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supernatant was equally divided into two tubes and incubated for 1 h on ice with 20 μl of 10% SA-conjugated with affinity-purified rabbit anti-myogenin antibody (Hashimoto et al., submitted) or anti-GTPase-activating protein (GAP) C-terminal peptide antibody as a control [31]. After centrifugation, the pellets were washed 3 times with RIPA buffer and then boiled in two-times concentrated SDS (2 × SDS) sample buffer. Labeled proteins were resolved by 15% SDS polyacrylamide gel electrophoresis (acrylamide : methylene bis-acrylamide = 300 : 1.7) and electrotransferred to polyvinylidine difluoride membranes (Trans-Blot, Bio-Rad). Labeled proteins were detected with a Fuji Image Analyzer BAS 2000 and then membranes were probed with anti-myogenin antibody followed by probing with a swine anti-rabbit IgG conjugated with alkaline phosphatase (Dako) as described elsewhere (Hashimoto et al., submitted).

2.3. In vitro transcription and translation

Rat myogenin cDNA [7] was subcloned into the *Eco*RI site of Bluescript II (Stratagene). Myogenin mRNA was transcribed in vitro using 2 μg of linearized plasmid according to the manufacturer's recommendations (Stratagene, mRNA Capping Kit). One μg of myogenin mRNA was translated with rabbit reticulocyte lysate in a 50-μl reaction volume containing [^{35}S]cysteine (ICN Radiochemicals) or cold cysteine according to the manufacturer's recommendations (Promega).

2.4. In vitro phosphorylation

[^{35}S]Cysteine-labeled myogenin (3 μl) was phosphorylated by incubation at 30°C for up to 60 min in a 10-μl reaction mixture containing 50 mM Tris-HCl, pH 8.0, 50 mM sodium β-glycerophosphate, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EGTA, 1 mM ATP, and purified cyclin B/CDC2 complex. The reaction was terminated by adding 2.5 μl of 5 × SDS sample buffer. Labeled myogenin was resolved by SDS-PAGE and detected with an Image Analyzer. The cyclin B/CDC2 complex was purified from starfish oocytes as described previously [32,33]. Briefly, starfish oocytes perfectly synchronized at the first meiotic metaphase were crushed and the cyclin B/CDC2 complex was purified from a soluble fraction by a p13^{suc1}-affinity column chromatography. The preparation does not contain cyclin A/CDC2 complex.

The following synthetic peptides were used as substrates for in vitro phosphorylation: MP3b (RRHSASCSPEWGNALE), Kemptide (cAMP-dependent protein kinase substrate, LRRASLG), and casein kinase II substrate (RRREEETEEE). Active CDC2 kinase was incubated with 500 μM peptide at 30°C for up to 60 min in the presence of 50 μM ATP (1 μCi of [^{32}P-γ]ATP/10 μl reaction volume). After incubation, 8 μl of reaction mixture was spotted on phosphocellulose paper, washed 3 times with 1% phosphoric acid, and air-dried. The radioactivity on the filter was measured with a liquid scintillation counter in the presence of scintillator.

2.5. Chemical cleavage and phosphopeptide analysis

Myogenin translated in vitro (100 μl) was phosphorylated for 1 h as described above in a 200 μl reaction volume containing 100 μCi [γ-^{32}P]ATP (New England Nuclear, Radiochemicals, 4500 Ci/mmol). The reaction was terminated by adding 4 μl of 2.5 mM staurosporine (Sekagakukougyou, Tokyo). Myogenin was immunoprecipitated with anti-myogenin antibody as described. Immunoprecipitates from 5 independent reactions were pooled, separated on SDS-PAGE, and transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel). Phosphorylated proteins detected with an Image Analyzer were excised and cleaved by *N*-chlorosuccinimide essentially as described by Lischwe and Sung [34]. The cleaved peptides were lyophilized, dissolved in peptide gel sample buffer (4% SDS, 50 mM Tris-HCl, pH 6.8, 50% glycerol, 2% β-mercaptoethanol), and boiled for 5 min. Peptides were separated on Tricine-SDS-PAGE ([35], ACI Japan Co., Kanagawa).

2.6. Site-directed mutagenesis

Site-directed mutagenesis was performed with single-stranded DNA templates according to the manufacturer's recommendations (Stratagene, Mutator DNA polymerase III site-directed mutagenesis kit). Mutants were generated with the following oligonucleotides identical to the antisense chain:

S7A, CTG, GTA, GAA, ATA, GGG, GGC, TGT, TTC, ATA, CAG, CTC
S43G, CCC, TCG, GGC, TTC, CGG, GCC, TAA, GCT, GAG, CTC
T57A, TGG, ACA, ATG, CTC, AGG, GGC, CCC, CAG, TCC, CTT
S170A, ATT, GCC, CCA, CTC, CGG, TGC, GCA, GGA, GGC, GCT

All mutations and coding regions included in the templates were confirmed by DNA sequencing.

3. Results

3.1. Myogenin is phophorylated at multiple sites during myogenesis

Myogenin was detected as a pair of bands during the myogenesis of C2C12 cells by immunoblotting (Fig. 1, lane 4) and the slow migrating band on SDS-PAGE is assumed to be phosphorylated form [22]. However, direct evidence for the phosphorylation of myogenin during myogenesis has not been reported. Thus, C2C12 myotubes were labeled with [^{32}P]orthophosphate and the cellular myogenin was immunoprecipitated with an affinity purified anti-myogenin antibody (Hashimoto et al., submitted). Immunoprecipitates were separated on SDS-PAGE and then electrotransferred to PVDF membranes. A pair of phosphorylated bands was detected with an Image Analyzer (Fig. 1, lane 2) and the membrane was probed with anti-myogenin antibody (Fig. 1, lane 4). The electrophoretic mobility of a pair of phosphorylated bands (Fig. 1, lane 2) was identical to that of the myogenin band pair (Fig. 1, lane 4). The results clearly show that both the slow migrating form and the fast migrating form of myogenin are phosphorylated in vivo. The protein ratio of the fast-to-slow migrating forms was approximately 4 : 1 whereas their ratio of phosphate incorporation was approximately 1 : 1. Thus, the slow migrating band is a hyperphosphorylated form.

3.2. CDC2 kinase phosphorylates myogenin and reduces its electrophoretic mobility in vitro

PKA [21] and PKC [20] have been reported to phosphorylate myogenin, although neither kinase mimics the electrophoretic mobility-shift that occurs in vivo. CDC2 kinase is another candidate for the modification of myogenin that results in the reduction of electrophoretic mobility because myogenin con-

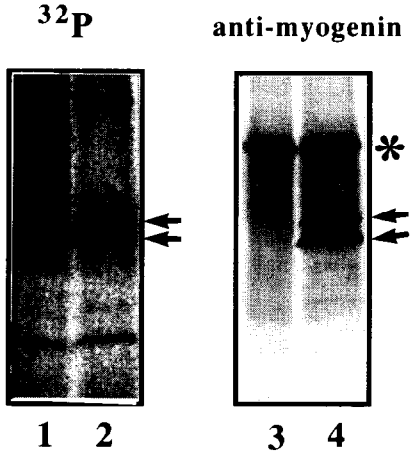

Fig. 1. Phosphorylation of myogenin in myotubes. Myogenin immunoprecipitates from ^{32}P-labeled C2C12 myotube lysates were separated on SDS-PAGE, and electrotransferred to a PVDF membrane. After the detection of radioactivity (lanes 1 and 2), myogenin was detected with affinity purified anti-myogenin antibody (lanes 3 and 4). Lanes 1 and 3, immunoprecipitates with anti-rasGAP antibody as an unrelated antibody. Lanes 2 and 4, immunoprecipitates with anti-myogenin antibody. Asterisk, heavy chain of immunoglobulin used for immunoprecipitation. Arrows represent myogenin as a pair of bands.

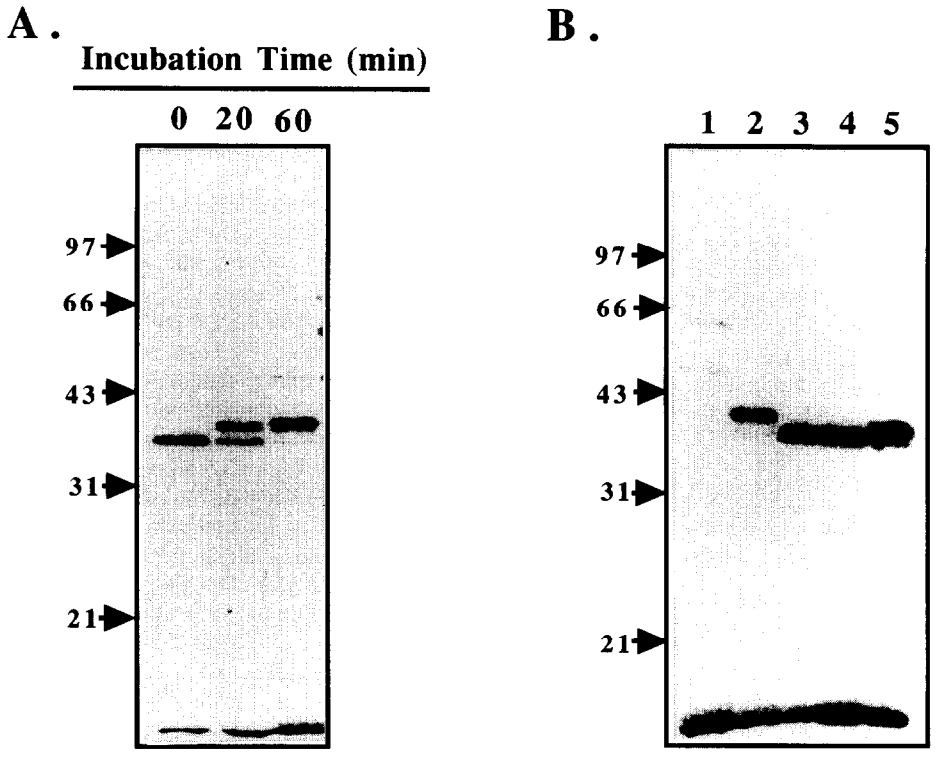

Fig. 2. The reduction of electrophoretic mobility of myogenin through in vitro phosphorylation by CDC2 kinase. (A) Reduction in the electrophoretic mobility of myogenin as a function of incubation time with active CDC2 kinase. ^{35}S-Labeled myogenin was phosphorylated by CDC2 kinase in vitro for the indicated periods and analyzed on SDS-PAGE. (B) Repression of the electrophoretic mobility-shift of myogenin by protein kinase inhibitors. Phosphorylation was carried out at 30°C for 60 min. Reaction mixtures contained: lane 1, reticulocyte lysate without myogenin RNA, CDC2 kinase, and 1 mM ATP; lane 2, reticulocyte lysate with myogenin RNA, CDC2 kinase, and 1 mM ATP; lane 3, reticulocyte lysate with myogenin RNA and 1 mM ATP; lane 4, reticulocyte lysate with myogenin RNA, CDC2 kinase, 1 mM ATP, and 50 μM staurosporine; lane 5, reticulocyte lysate with myogenin RNA, CDC2 kinase, 1 mM ATP, and 1 mM 6-dimethylaminopurine. The positions of molecular weight markers are shown on the left.

tains four serine-proline (SP) and threonine-proline (TP) sequences as possible phosphorylation sites for CDC2 kinase [36,37]. CDC2 kinase has been reported to reduce the electrophoretic mobility of neurofilaments on SDS-PAGE [33]. Thus, [^{35}S]cysteine-labeled myogenin produced by in vitro translation was incubated with active CDC2 kinase in the presence of 1 mM ATP and analyzed on SDS-PAGE. The electrophoretic mobility of myogenin was reduced from 36 kDa to 38 kDa in an incubation time-dependent manner (Fig. 2A). The reduction of electrophoretic mobility was not due to myogenin modification by reticulocyte lysate components because the electrophoretic mobility was not reduced in the absence of CDC2 kinase (Fig. 2B, lane 3). Furthermore, the electrophoretic mobility-shift was completely inhibited by protein kinase inhibitors, 50 μM staurosporine (Fig. 2B, lane 4) and 1 mM 6-dimethylaminopurine (Fig. 2B, lane 5). Thus, the electrophoretic mobility-shift is due to phosphorylation by CDC2 kinase.

3.3. *Phosphorylation at a proline-directed kinase motif is essential for the modification of myogenin in vivo*

CDC2 kinase is well known to be a proline-directed kinase (reviewed in [36,37]) and SP or TP motifs are candidates for phosphorylation by CDC2 kinase. There are four SP and TP sites in the rat myogenin polypeptide, including serine at residue number 7 (S7), serine at residue number 43 (S43), threonine at residue number 57 (T57), and serine at residue number 170 (S170). To examine which of these proline-directed kinase motifs are responsible for the reduction in electrophoretic mobility of myogenin in vivo, the four amino acids at the putative phosphorylation sites were replaced with non-phosphorylating amino acids by site-directed mutagenesis in the myogenin cDNA as follows: S7 to alanine (A7), S43 to glycine (G43), T57 to alanine (A57), and S170 to alanine (A170). The mutant

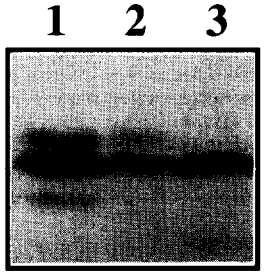

Fig. 3. Proline-directed kinase motif-dependent modification of myogenin in vivo. Mutant myogenin cDNA designed to encode a protein lacking all four SP/TP sites was constitutively expressed in 10T1/2 cells. Cell clones expressing wild type myogenin (clone G5 in lane 2) or mutant myogenin lacking SP/TP sites (clone GN21 in lane 3) were analyzed. C2C12 myotube lysate was analyzed as a control (lane 1). Thirty μg of cellular protein was separated on SDS-PAGE and processed for immunoblotting.

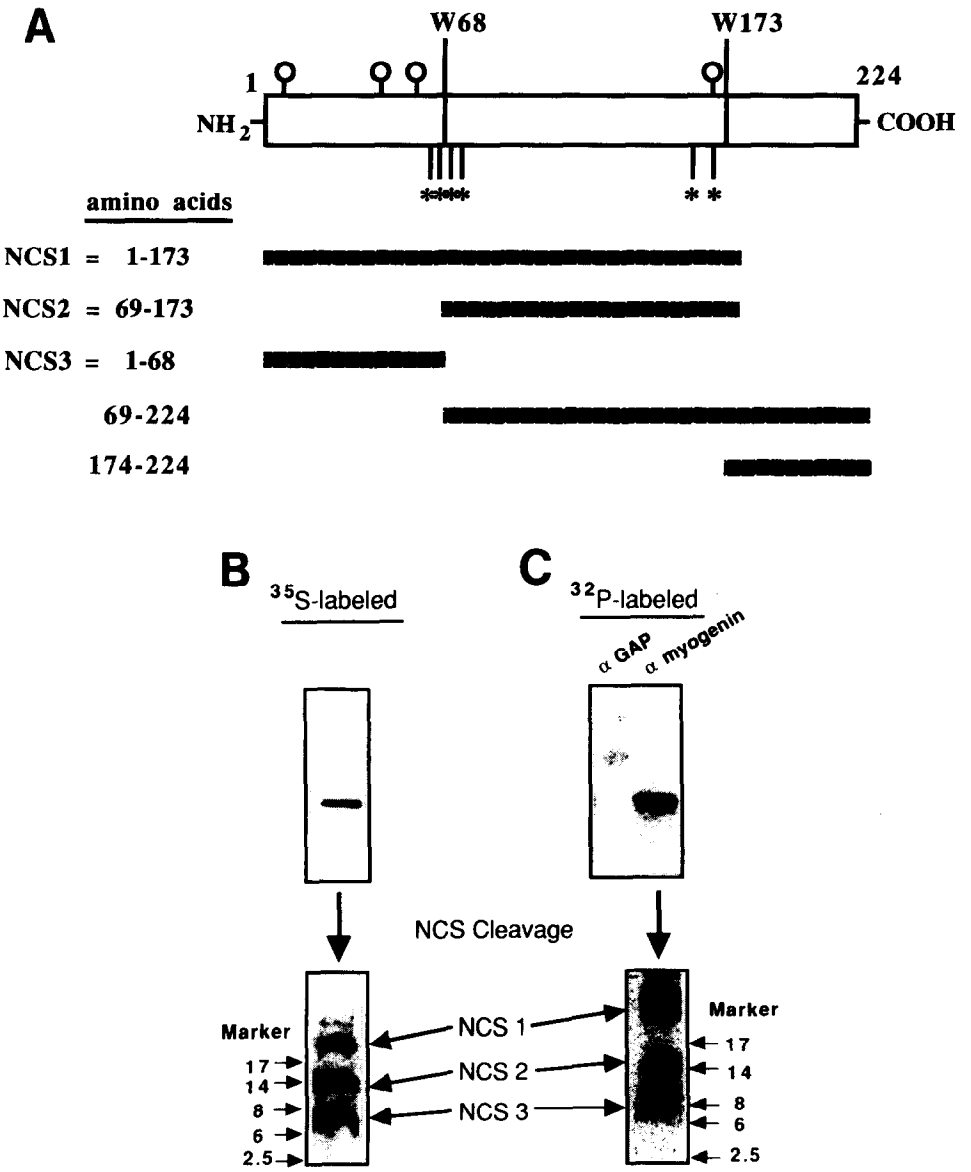

Fig. 4. One-dimensional phosphopeptide mapping of myogenin phosphorylated in vitro by CDC2 kinase. (A) Expected myogenin peptide fragments from *N*-chrolosuccinimide (NCS) cleavage. Striped bars represent possible fragments excised. NCS1, -2, and -3 correspond to the fragments shown in panels (B) and (C). W, positions of tryptophan residues; asterisks, positions of cysteine residues; circles, positions of SP/TP motifs. (B) [^{35}S]Cysteine-labeled myogenin was immunoprecipitated, separated, and electrotransferred to a nitrocellulose membrane. The labeled myogenin band was processed for NCS cleavage. Peptides were separated on Tricine-SDS-PAGE. (C) After incubation with CDC2 kinase in the presence of [γ-^{32}P]ATP, myogenin was immunoprecipitated, separated, and electrotransferred to a nitrocellulose membrane. The ^{32}P-labeled band specific for anti-myogenin antibody immunoprecipitates [compared to immunoprecipitates with unrelated antibody (anti-GAP antibody)] was analyzed as described in (B). The positions of molecular weight markers are shown on the left in panel (B) and the right in panel (C).

myogenin cDNA designed to encode myogenin lacking the four SP/TP motifs (A7G43A57A170) was expressed constitutively in 10T1/2 cells. We established several cell lines with various expression levels of myogenin and determined the patterns of wild type or mutant myogenin (A7G43A57A170) by immunoblotting. In contrast to wild type myogenin constitutively expressed in 10T1/2 cells (Fig. 3, lane 2), SP/TP motif-deficient myogenin was detected as a single band of 36 kDa on SDS-PAGE (Fig. 3, lane 3). This strongly suggests that phosphorylation of the SP/TP motif in myogenin in vitro by CDC2 kinase does in fact mimic the modification of myogenin in vivo that causes the reduction of electrophoretic mobility.

3.4. Phosphorylation of serine-43 by CDC2 kinase is responsible for the reduction in the electrophoretic mobility of myogenin

To identify sites in myogenin phosphorylated by CDC2 kinase, the peptide pattern of myogenin translated in vitro was analyzed by chemical cleavage with *N*-chlorosuccinimide (NCS). [^{35}S]Cysteine-labeled myogenin was immunoprecipitated by anti-myogenin antibody, separated on SDS-PAGE, and transferred to a nitrocellulose membrane (Fig. 4B, upper panel). Myogenin was detected with an Image Analyzer and a strip containing myogenin band was removed and processed for NCS cleavage as described in section 2. NCS cleaves polypeptides on the carboxyl side of tryptophan residues [34]. Rat

myogenin contains two typtophan residues at positions 68, 173 and thus cleaves into peptides containing amino acids 1–68, 69–173, and 174–224 (Fig. 4A). The expected [^{35}S]cysteine-labeled fragments from myogenin are peptides containing amino acids 1–173, 69–173, 1–68, and 69–224 because myogenin contains 6 cysteine residues at positions 61, 65, 70, 73, 162, and 169 (Fig. 4A). The apparent molecular weight of the labeled fragments obtained were 19 kDa, 14 kDa, and 7 kDa, and these were termed NCS1, NCS2, and NCS3, respectively (Fig. 4B). NCS2 and NCS3 were identified as the myogenin peptides comprising amino acids 69–173 and 1–68, respectively, from their apparent molecular sizes. NCS1 was believed to be the peptide comprising amino acids 1–173. However, we could not exclude the possibility that NCS1 corresponds to a peptide containing amino acids 69–224 because the apparent molecular size of peptide on electrophoresis is sometimes inconsistent with the estimated value. To identify phosphorylated peptides, myogenin translated in vitro was phosphorylated by CDC2 kinase in the presence of [γ-^{32}P]ATP and then immunoprecipitated with anti-myogenin antibody (Fig. 4C, right lane in the upper panel). Phosphopeptide analysis showed all of the NCS fragments were phosphorylated by CDC2 kinase (Fig. 4C). Therefore, multiple amino acids in myogenin are phosphorylated by CDC2 kinase and these sites reside in the regions comprising amino acids 1–68 (NCS3) and 69–173 (NCS2).

In order to determine the CDC2 kinase phosphorylation sites, three of the four amino acids at the putative phosphorylation sites were replaced with non-phosphorylating amino acids by site-directed mutagenesis. Complementary DNA encoding wild type or mutant myogenin was used as a template for in vitro transcription and then wild type and mutant myogenins were produced by in vitro translation. Mutant myogenin proteins except A7G43A57A170 were designed to contain a single proline-directed kinase motif. Wild type or mutant myogenin was incubated with active CDC2 kinase in the presence of ATP, immunoprecipitated with anti-myogenin antibody, and transferred to PVDF membranes. The membranes were then probed with anti-myogenin antibody. The electrophoretic mobility of myogenin on SDS-PAGE was reduced only in wild type and A7A57A170, whereas the mobilities of the other mutants (A7G43A57A170, G43A57A170, A7G43A170, and A7G43A57) did not change (Table 1). The phosphorylation site resulting in the reduction of electrophoretic mobility of myogenin was shown clearly to be S43.

Table 1
Electrophoretic mobility of mutant myogenin after phosphorylation by CDC2 kinase in vitro

Mutant name	Positions of amino acids				Band-shift
	7	43	57	170	
Wild type	S	S	T	S	+
A7G43A57A170	A	G	A	A	−
G43A57A170	S	G	A	A	−
A7A57A170	A	S	A	A	+
A7G43A170	A	G	T	A	−
A7G43A57	A	G	A	S	−

Wild type and mutant myogenin were incubated with active CDC2 kinase in the presence of 1 mM ATP and then separated on SDS-PAGE. The presence (+) or absence (−) of the slow migrating form of myogenin was determined.

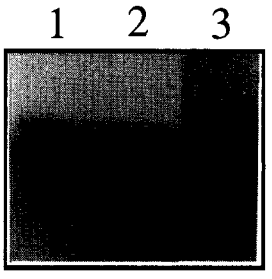

Fig. 5. Reduction of electrophoretic mobility dependent on S43 in myogenin in vivo. Wild type or mutant myogenin in which S43 was replaced by glycine (G43) was transiently expressed in COS-7 cells. Myogenin was detected by immunoblotting. Fifty μg of cellular protein was analyzed. Lane 1, C2C12 myotubes; lane 2, wild type myogenin expressing COS-7 cells; lane 3, the S43 mutant expressing COS-7 cells.

3.5. *The in vitro CDC2 kinase phosphorylation site is essential for the modification of myogenin in vivo*

In order to demonstrate that S43 is the essential amino acid for the reduction of electrophoretic mobility in living cells, a mutant myogenin cDNA designed to replace S43 with glycine (G43) was constructed and transiently expressed in COS-7 cells. Myogenin was detected as a pair of bands in wild type myogenin-expressing COS-7 cells (Fig. 5, lane 2) as shown in C2C12 cells. In contrast, only the fast migrating form of myogenin was present in G43-expressing COS-7 cells (Fig. 5, lane 3). Thus, S43 appears to be essential for the modification of myogenin in vivo that results in the reduction in electrophoretic mobility through phosphorylation.

3.6. *Serine-170 is another candidate CDC2 kinase phosphorylation site in vitro*

Phosphopeptide mapping showed another CDC2 kinase phosphorylation site in NCS2 corresponding to the region comprising amino acids 69–173 (Fig. 4C). This fragment includes a single proline-directed kinase phosphorylation site, S170. We found that a β-galactosidase myogenin carboxyl-terminal domain (amino acids 134–224) fusion protein is phosphorylated by CDC2 kinase in vitro (data not shown). Furthermore, a synthetic peptide comprising amino acids 165–178 of myogenin, MP3b, is a suitable substrate for CDC2 kinase. Active CDC2 kinase phosphorylates an SP motif-containing peptide, MP3b, very efficiently whereas the serine or threonine residues in Kemptide or casein kinase II substrate peptide were not phosphorylated (Fig. 6). The results suggest that CDC2 kinase phosphorylates peptides in a proline-directed kinase motif-dependent manner. Thus, S170 is assumed to be another CDC2 kinase phosphorylation site in vitro, which is not responsible for the electrophoretic mobility-shift of mytogenin (Table 1).

4. Discussion

It has been widely reported that the function of transcription factors is directly regulated by phosphorylation (reviewed in [38]). MyoD family members are skeletal muscle-specific transcription factors reported to be phosphoproteins [22,39,40]. Extracellular signaling molecules, including bFGF, TGFb, and serum, but not insulin, suppress the function of myogenic determining factors (reviewed in [10]), presumably through phosphorylation [20,41]. The DNA binding activity of myogenin is

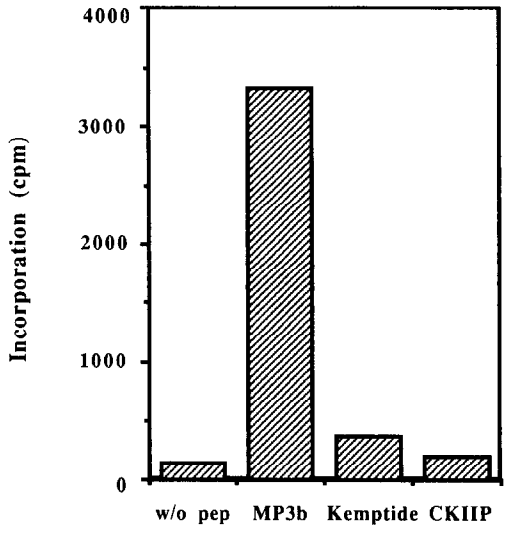

Fig. 6. Phosphorylation of myogenin peptide by CDC2 kinase in vitro. Synthetic peptides were incubated with active CDC2 kinase in the presence of [γ-^{32}P]ATP at 30°C for 60 min. Reaction mixtures contained no peptide substrate (w/o pep) or 500 μM of the indicated peptide. MP3b, the peptide comprising amino acids 165–178 of myogenin; Kemptide, cAMP-dependent protein kinase substrate peptide; CKIIP, casein kinase II substrate peptide. Averages of duplicate samples are shown.

repressed through phosphorylation of its basic region by PKC, a downstream component of bFGF in non-myogenic cells [20]. Overexpressed myogenin in COS cells is phosphorylated by PKA but myogenin function is not affected [21]. Here we provide direct evidence that myogenin undergoes multiple phosphorylation during myogenesis, although previous studies report the phosphorylation of myogenin in proliferating non-myogenic cells. The present results imply that myogenin function is regulated through phosphorylation during myogenesis.

Furthermore, we found that phosphorylation of S43 is responsible for the reduction in the electrophoretic mobility of myogenin in vivo as well as in vitro. This phosphorylation would cause a significant change in the protein structure of myogenin and affect its function. Schwarz et al. [42] showed that the amino-terminal domain including S43 is important for the transcriptional activity of myogenin. Thus, it is conceivable that phosphorylation of S43 negatively regulates the transcriptional activity of myogenin although S43 phosphorylation does not suppress its DNA binding activity (Hashimoto, unpublished). Functional changes in myogenin by S43 phosphorylation are currently under investigation in our laboratory.

In addition to S43, here we propose S170 as another possible phosphorylation site by CDC2 kinase in vitro (Fig. 4). S170 resides in the carboxyl terminal activation domain of myogenin [42]. The amino acid sequence around S170, SCSPEW, is conserved among avian and mammalian myogenins [43] whereas the other three SP/TP motifs are present only in mammals. We currently investigate the role of S170 phosphorylation in the regulation of myogenin function including suppression of its transcriptional activity. We are also pursuing the possibility that S7 and T57 in other SP/TP motifs undergo phosphorylation during myogenesis.

In vitro system, CDC2 kinase modifies myogenin by phosphorylating S43, a process that also occurs in vivo. Hyperphosphorylated myogenin (the S43-phosphorylated form) is present throughout myogenesis although CDC2 kinase is promptly down-regulated in differentiating myoblasts ([44,45], Hashimoto, unpublished). Thus, another proline-directed kinase would phosphorylate S43 in vivo. Possible candidates are members of the CDC2 family and mitogen-activated protein (MAP) kinases because these kinases sometimes phosphorylate the same protein sites as CDC2 kinase [37]. Our mutant myogenin would be useful in identifying the myogenin kinase responsible for the phosphorylation of S43, assumed to be a negative regulator of myogenin. Phosphorylation of proline-directed kinase motifs is also reported in cytoskeletal proteins from striated muscle, dystrophin [46] and titin [47]. Proline-directed kinases would play multiple roles in myogenic differentiation through phosphorylation of various molecules. There is no doubt that the phosphorylation of myogenin plays a critical role in myogenic differentiation.

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