Biochemical and Biophysical Research Communications 433 (2013) 558–562

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Requirement of decreased *O*-GlcNAc glycosylation of Mef2D for its recruitment to the *myogenin* promoter

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ARTICLE INFO

Article history:
Received 4 March 2013
Available online 22 March 2013

Keywords:
O-GlcNAc
Glycosylation
Mef2D
Myogenesis
Myogenin

ABSTRACT

Previously, we demonstrated that the expression of *myogenin*, a critical transcription factor for myogenesis, is negatively regulated by *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) glycosylation in mouse C2C12 cells. In this study, we found that Mef2 family proteins, especially Mef2D which is a crucial transcriptional activator of *myogenin*, are *O*-GlcNAc glycosylated. Between the two splice variants of Mef2D, Mef2D1a rather than Mef2D1b appears to drive the initiation of *myogenin* expression in the early stage of myogenesis. A deletion mutant analysis showed that Mef2D1a is glycosylated both in its DNA-binding and transactivation domains. A significant decrease in the glycosylation of Mef2D was observed in response to myogenic stimulus in C2C12 cells. Inhibition of the myogenesis-dependent decrease in the glycosylation of Mef2D suppressed its recruitment to the *myogenin* promoter. These results indicate that the expression of *myogenin* is regulated, at least in part, by the decreased glycosylation-dependent recruitment of Mef2D to the promoter region, and this is one of the negative regulatory mechanisms of skeletal myogenesis by *O*-GlcNAc glycosylation.

1. Introduction

O-linked β-*N*-acetylglucosamine (*O*-GlcNAc) glycosylation on the Ser/Thr residues of nuclear, cytosolic, and mitochondrial proteins is a dynamic post-translational modification found in the multicellular organisms [1,2]. Its cycling is regulated by *O*-GlcNAc transferase and *O*-GlcNAcase and more than 1000 proteins have now been identified to be *O*-GlcNAc glycosylated [1,2]. *O*-GlcNAc glycosylation is thought to be involved in various cellular functions including transcription, epigenesis, cellular signaling, stress response, and glucose sensing [1,2]. In addition, it has been focused on a cell lineage-dependent functional significance of *O*-GlcNAc glycosylation on differentiation [3–9].

We previously demonstrated that the terminal differentiation program of skeletal myogenesis of mouse C2C12 cells is negatively regulated by *O*-GlcNAc glycosylation [10]. The global decrease in *O*-GlcNAc glycosylation was observed at the earlier stage of skeletal myogenesis [10]. The decrease in the glycosylation was required for the expression of *myogenin*, a critical transcription factor for myogenesis, and myotube formation [10]. Yet, the negative regulatory mechanism of *myogenin* expression by the glycosylation has not been clarified.

Here, we demonstrate that Mef2D, a crucial transcriptional activator of *myogenin* expression [11], is *O*-GlcNAc glycosylated both in its DNA-binding and transactivation domains. A significant decrease in the glycosylation of Mef2D was observed in response to myogenic stimulus. Furthermore, we show that inhibition of myogenesis-dependent decrease in the glycosylation of Mef2D suppresses its recruitment to the *myogenin* promoter. These results indicate that the expression of *myogenin* is regulated, at least in part, by the decreased glycosylation-dependent recruitment of Mef2D to *myogenin* promoter.

2. Materials and methods

2.1. Materials

Anti-*O*-GlcNAc (CTD110.6) antibody was a kind gift from Dr. Gerald W. Hart (Johns Hopkins University School of Medicine). Anti-β-actin (I-19), anti-lamin B (M-20), anti-Mef2 (C-21), anti-MyoD (C-20), and normal mouse IgG antibodies were obtained from Santa Cruz Biotechnology. Anti-Flag (M2) and anti-α-tubulin antibodies were obtained from Sigma–Aldrich and anti-*O*-GlcNAc antibody (RL2) was from Thermo Scientific. Anti-Mef2D antibody (9/MEF2D) was obtained from BD Transduction Laboratories. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG and HRP-anti-rabbit IgG antibodies were obtained from GE Healthcare, and HRP-anti-goat IgG antibody was from R&D Systems. Thiamet G was obtained from Cayman Chemical.

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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.03.033

2.2. Cell culture

Mouse C2C12 myoblasts and human embryonic kidney 293T cells were cultured in DMEM containing 10% FBS. C2C12 myoblasts were differentiated as described previously [10]. Briefly, cells were plated at a density of 3×10^5 cells/9 cm^2. After 24 h, the medium was replaced by DMEM containing 2% horse serum.

2.3. Cloning, site-directed mutagenesis, and transfection

A mouse cDNA library was prepared from C2C12 myoblasts or C2C12 myotubes, and mouse Mef2A, Mef2C, Mef2D1a, Mef2D1b and MyoD were subcloned into a mammalian expression vector, pcDNA3.1-His-Flag-Flag [12], using In-Fusion HD Cloning kit (Takara Bio). Deletion mutants of Mef2D1a were generated by site-directed mutagenesis using PrimeSTAR Mutagenesis Basal kit (Takara Bio). All constructs were confirmed by DNA sequencing. Each expression vector was transiently transfected into 293T cells using Lipofectamine 2000 transfection reagent (Life Technologies). All primers used are shown in Supplementary Table 1.

2.4. Immunoprecipitation and Western blotting

For immunoprecipitation, cells were lysed in 50 mM Tris–HCl (pH 7.4), 70 mM 2-mercaptoethanol and 0.5% SDS, boiled for 5 min, and diluted with 4 volumes of 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.25% Triton ×-100, and protease inhibitors cocktail (Nacalai Tesque). The lysates were added with an appropriate antibody and incubated for 2 h at 4 °C. The immune complexes were precipitated with protein A/G PLUS-agarose (Santa Cruz Biotechnology) and washed extensively with 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 0.25% Triton ×-100. Western blotting was performed as described previously [10].

2.5. Biotin labeling and purification of O-GlcNAc glycosylated proteins

O-GlcNAc residues of the glycoproteins were labeled with biotin-alkyne using Click-it *O*-GlcNAc Enzymatic Labeling System (Life Technologies) and Click-it Biotin Protein Analysis Detection kit (Life Technologies) according to the manufacturer's protocols. After labeling, the biotin-labeled glycoproteins were purified with Soft Release Avidin-Resin (Promega).

2.6. Chromatin immunoprecipitation (ChIP)

Approximately 3×10^6 cells were cross-linked with a 1% final concentration of formaldehyde for 20 min at 37 °C. ChIP assays were performed with EZ-ChIP kit (Millipore) according to the manufacturer's instruction. ChIP-purified DNA was amplified by standard PCR using specific primers for the *myogenin* promoter region (Supplementary Table 1). After amplification, PCR products were separated on 3% agarose gels and visualized by ethidium bromide fluorescence.

3. Results and discussion

3.1. Mef2D is modified by O-GlcNAc glycosylation

We have previously demonstrated that inactivation of *O*-GlcNAcase inhibits the myogenesis-dependent expression of *myogenin* in mouse C2C12 cells [10]. To address the inhibitory mechanism of the *myogenin* expression, we first examined whether the transcription factors of *myogenin*, including MyoD and Mef2 family proteins, are modified by *O*-GlcNAc glycosylation. Flag-tagged MyoD or Mef2 family proteins transiently expressed in 293T cells in the absence or the presence of thiamet G, a very potent and highly specific inhibitor of *O*-GlcNAcase [13], were immunoprecipitated and analyzed by immunoblotting using two kinds of anti-*O*-GlcNAc antibodies, CTD110.6 and RL2 (Fig. 1A). It has been shown that both CTD110.6 and RL2 require *O*-GlcNAc as part of their epitopes, and that they show different specificities [14]. Flag-Mef2D1a and Flag-Mef2D1b were clearly detected by immunoblotting with both anti-*O*-GlcNAc antibodies, and the immunoreactivities of both proteins were significantly increased in 293T cells cultured in the presence of thiamet G. The immunoreactivities of both anti-*O*-GlcNAc antibodies were specifically competed away in the presence of 0.1 M GlcNAc. Flag-Mef2A and Flag-Mef2C were slightly detected by immunoblotting with CTD110.6, but could hardly with RL2. In the same condition, Flag-MyoD was not detectable with both antibodies. To further confirm *O*-GlcNAc glycosylation, we applied a chemoenzymatic approach to detect terminal GlcNAc residues [15]. Terminal GlcNAc residues of glycoproteins in cell lysates of 293T cells, which were transiently expressed with Flag-tagged MyoD or Mef2 family proteins, were specifically labeled with azido-modified galactose by mutant β-1,4-galactosyltransferase (GalT) followed by a biotin-alkyne tag addition. In a negative control experiment, the reaction was performed in the absence of GalT (ΔGalT). The labeled glycoproteins with biotin were purified and analyzed by immunoblotting with anti-Flag antibody (Fig. 1B). Both Flag-Mef2D1a and Flag-Mef2D1b were strongly detected and both Flag-Mef2A and Flag-Mef2C were just detectable by the immunoblotting, but Flag-MyoD was not. These results indicate that transiently expressed Mef2 family proteins, including Mef2A, Mef2C, Mef2D1a, and Mef2D1b, are *O*-GlcNAc glycosylated in 293T cells.

The chemoenzymatic approach was used to examine *O*-GlcNAc glycosylation of endogenous Mef2 family proteins and MyoD in C2C12 myoblasts (Fig. 1C). Although Mef2A, Mef2C, and MyoD were not detectable with this approach, Mef2D was certainly identified as a glycoprotein which has the terminal GlcNAc residue. We next performed immunoblotting of endogenous Mef2D with anti-*O*-GlcNAc antibodies (Fig. 1D). Mef2D immunoprecipitated from C2C12 myoblasts was clearly detected by both CTD110.6 and RL2. These results indicate that endogenous Mef2D in C2C12 myoblasts is an *O*-GlcNAc glycosylated protein. Since the molecular masses of Mef2D1a and Mef2D1b are very close and the anti-Mef2D antibody which we used in this study reacts with both Mef2D1a and Mef2D1b, we could not distinguish the two splice variants.

3.2. Mef2D1a is the dominant splice variant of mef2d in the early stage of skeletal myogenesis

It has been shown that *mef2d* is alternatively spliced and expresses two variants; Mef2D1a and a muscle-specific isoform of Mef2D1b [16,17]. To characterize the alternative splicing status of *mef2d* during myogenesis, C2C12 myoblasts were induced to differentiate for 5 days and the expression levels of *mef2d1a* and *mef2d1b* were monitored at intervals of 24 h (Fig. 2A and B). While the expression levels of *mef2d1a* were constant throughout myogenesis, those of *mef2d1b* were up-regulated and were significantly increased on and after day 4. The expression levels of Mef2D, which include both Mef2D1a and Mef2D1b, were constant throughout myogenesis (Fig. 2C). This result indicates that Mef2D1a is the dominant splice variant of *mef2d* in the early stage of myogenesis and that the expression of Mef2D1b is required for the later stage. This is consistent with a previous report that Mef2D1b plays critical roles in the expression of muscle-specific genes such as *myosin heavy chain* and the myotube formation [18]. Since the expression of *myogenin* was detected on and after day 1 (Fig. 2B), Mef2D1a, rather than Mef2D1b, may participate

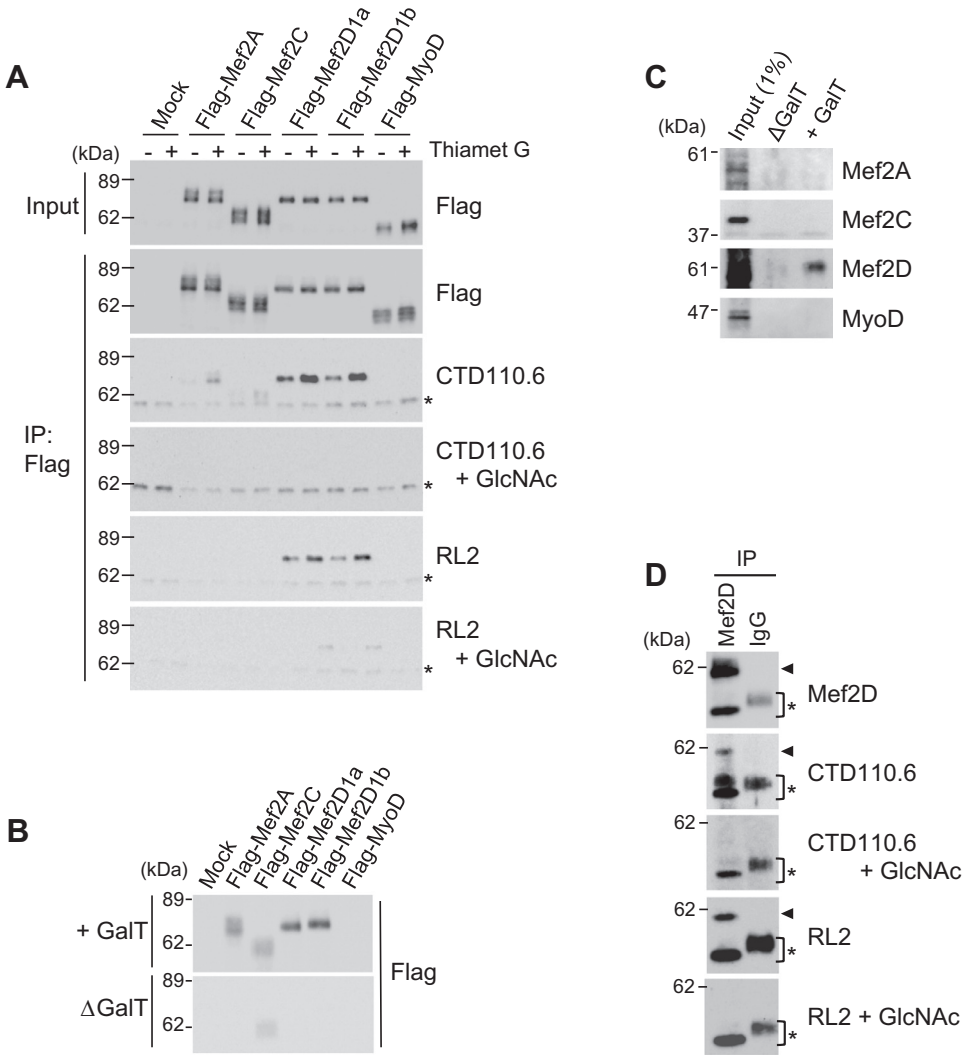

Fig. 1. Mef2D is modified by *O*-GlcNAc glycosylation. (A) Flag-tagged mouse Mef2 family proteins and MyoD were transiently expressed in 293T cells in the absence or the presence of 50 nM of thiamet G for 24 h and the expressed proteins were immunoprecipitated with anti-Flag antibody (IP: Flag). Representative Western blots of the immunoprecipitates probed with the indicated antibodies are shown. The specificity of the immunoreactivity with anti-*O*-GlcNAc antibodies (CTD110.6 and RL2) was confirmed by a hapten inhibition test using 0.1 M GlcNAc (+GlcNAc panels). Asterisks denote IgG heavy chain. (B) Flag-tagged mouse Mef2 family proteins and MyoD were transiently expressed in 293T cells and cell lysates were prepared. *O*-GlcNAc glycosylated proteins were specifically biotinylated and purified by streptavidin-resin. Representative Western blots of the biotinylated proteins probed with anti-Flag antibody are shown (+GalT panel). ΔGalT was a negative control experiment of biotinylation. (C) *O*-GlcNAc glycosylated proteins in C2C12 myoblasts were specifically biotinylated, purified by streptavidin-resin, and immunoblotted with the indicated antibodies (+GalT). ΔGalT was a negative control experiment of biotinylation. (D) Endogenous Mef2D was immunoprecipitated from C2C12 myoblasts and immunoblotted with the indicated antibodies. The specificity of immunoreactivities with anti-*O*-GlcNAc antibodies (CTD110.6 and RL2) was confirmed by a hapten inhibition test using 0.1 M GlcNAc (+GlcNAc panels). Arrowheads and asterisks denote Mef2D and IgG heavy chain, respectively. Normal mouse IgG (IgG) was used as a negative control experiment of immunoprecipitation.

in the initiation of *myogenin* expression in the early stage of myogenesis.

3.3. Mef2D1a is O-GlcNAc glycosylated both in its DNA-binding and transactivation domains

Mef2D1a contains a MADS-box and a MEF2 domain at the N-terminus, which together regulate dimerization and DNA binding, and the transcriptional activation domain at the C-terminus, which includes a beta domain and a Gln/Pro-rich region [11,16]. To define the region possessing *O*-GlcNAc glycosylation site on Mef2D1a, we used a series of Flag-tagged deletion mutants of Mef2D1a, which include Δ1–86, Δ87–132, Δ133–291, Δ292–402 and Δ403–514 (Fig. 3A). Wild type or deletion mutants of Mef2D1a transiently expressed in 293T cells were purified and immunoblotted with two anti-*O*-GlcNAc antibodies (Fig. 3B). Wild type Mef2D1a, Δ87–132, and Δ133–291 were clearly detected by immunoblotting with both anti-*O*-GlcNAc antibodies. Δ1–86 was detected only with RL2 and Δ403–514 was only with CTD110.6. Δ292–402 could hardly detect with both antibodies. This result indicates that Mef2D1a possesses multiple *O*-GlcNAc glycosylation sites both in its DNA-binding and transactivation domains and that the most prominent glycosylation site is located in aa 292–402 of the transactivation domain.

3.4. Decreased O-GlcNAc glycosylation of Med2D is required for myogenesis-dependent its recruitment to myogenin promoter

To gain insights into relevance of *O*-GlcNAc glycosylation of Mef2D for *myogenin* expression, we inhibited *O*-GlcNAc glycosylation of *Mef2D* and monitored its recruitment to the *myogenin* promoter. C2C12 myoblasts were induced to differentiate in the

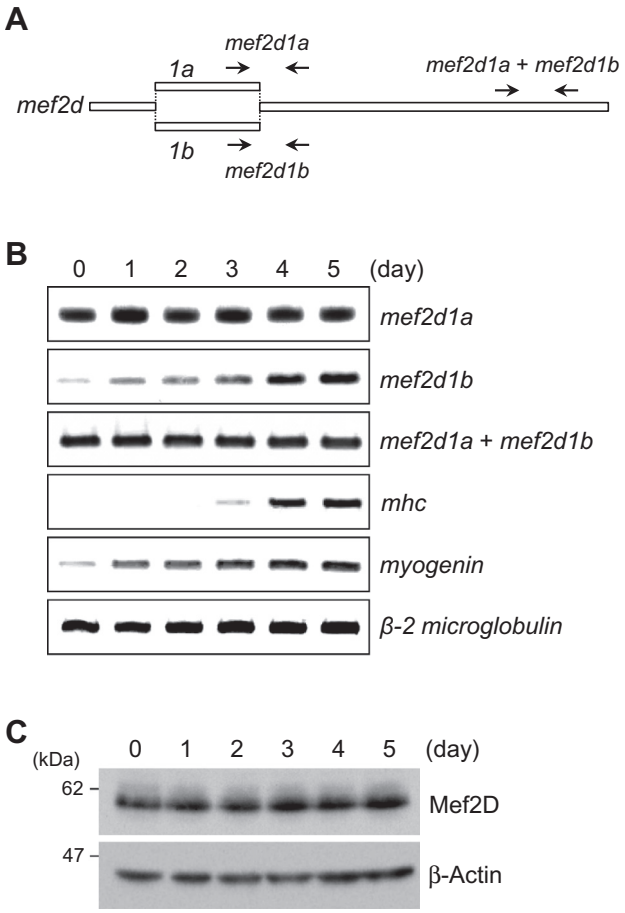

Fig. 2. Mef2D1a is the dominant splice variant of *mef2d* in the early stage of skeletal myogenesis. (A) A schematic diagram of two *mef2d* splice variants, *mef2d1a* and *mef2d1b*. Arrows indicate region for specific amplification of *mef2d1a*, *mef2d1b*, or both *med2d1a* and *mef2d1b*. (B) C2C12 myoblasts were induced to differentiate for 5 days. Representative RT-PCR analyses of the indicated genes are shown. *Mhc*, *myosin heavy chain*. (C) C2C12 myoblasts were induced to differentiate for 5 days. Expression levels of Mef2D were monitored by Western blotting. β-Actin was used loading control.

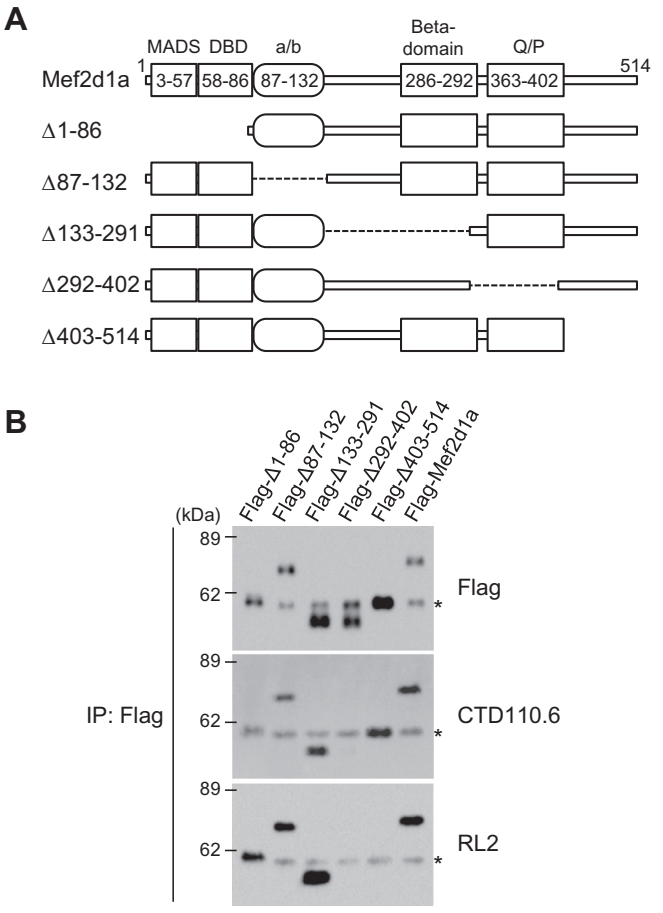

Fig. 3. Mef2D1a is *O*-GlcNAc glycosylated both in its DNA-binding and transactivation domains. (A) A schematic diagram of mouse Mef2D1a and a series of its deletion mutants, lacking 1–86 aa (Δ1–86), 87–132 aa (Δ87–132), 133–291 aa (Δ133–291), 292–402 aa (Δ292–402), and 403–514 aa (Δ403–514) residues. (B) Flag-tagged mouse Mef2D1a and its deletion mutants were transiently expressed in 293T cells and cell lysates were prepared. Representative Western blots of the immunoprecipitates with the indicated antibodies are shown. Asterisks denote IgG heavy chain.

absence or the presence of thiamet G, and the levels of *O*-GlcNAcylation of endogenous Mef2D were analyzed by the chemoenzymatic approach. Terminal GlcNAc residues of glycoproteins in cell lysates of C2C12 cells were labeled with biotin, and the labeled glycoproteins were purified and analyzed by immunoblotting with anti-Mef2D antibody. As shown in Fig. 4A, *O*-GlcNAc glycosylation levels of Mef2D decreased significantly by the induction of differentiation (-thiamet G). In the presence of thiamet G, the myogenesis-dependent decrease in *O*-GlcNAcylation of Mef2D was clearly suppressed. Thiamet G did not affect the expression levels of Mef2D during myogenesis (data not shown).

Mef2D has been shown to be recruited to the *myogenin* promoter in response to the myogenic induction [19]. We analyzed the effect of thiamet G on the myogenesis-dependent recruitment of Mef2D to the *myogenin* promoter using chromatin immunoprecipitation assay. A schematic diagram of mouse *myogenin* promoter is shown in Fig. 4B. The promoter region (−154 to −8), which includes both MEF2-binding site (M) and E-boxes (E1 and E2), and the distal region (−1565 to −1447) were amplified with specific primers, respectively. A specific recruitment of Mef2D to the promoter region was significantly increased in response to myogenic induction, and it was clearly suppressed by thiamet G treatment (Fig. 4C). The specificity of the *myogenin* promoter region-specific recruitment of Mef2D was confirmed by both the immunoprecipitation using normal mouse IgG and the amplification of the distal region. This result indicates that the myogenesis-dependent decrease in *O*-GlcNAc glycosylation of Mef2D promotes its recruitment to the *myogenin* promoter. Interestingly, it has been reported that the DNA-binding activity of Mef2 is reduced by the mutation of Thr^{20} and Ser^{59}, a known phosphorylation sites of Mef2 [20,21]. In this study we demonstrated that the DNA-binding domain of Mef2D1a including Thr^{20} and Ser^{59} possesses potent *O*-GlcNAc glycosylation site(s). *O*-GlcNAc glycosylation of the DNA-binding domain of Mef2D may inhibit phosphorylation of Thr^{20} and Ser^{59} to reduce its DNA-binding activity. Further study will be necessary to identify the *O*-GlcNAc glycosylation site(s) on Mef2D and to clarify the inhibitory mechanism of the DNA-binding activity of Mef2D by glycosylation. In addition, the effect of *O*-GlcNAc glycosylation on transcriptional activity of Mef2D is another issue to be clarified since the most prominent glycosylation site(s) of Mef2D1a is located in the transactivation domain as we showed in this study.

In summary, our finding shows that *O*-GlcNAc glycosylation has an inhibitory role of Mef2D, a crucial transcriptional activator of *myogenin* expression, and that the myogenesis-dependent decrease in the glycosylation of Mef2D enables its recruitment to the *myogenin* promoter. Therefore, the terminal differentiation program of the skeletal myogenesis is negatively regulated at least in part by *O*-GlcNAc glycosylation of Mef2D.

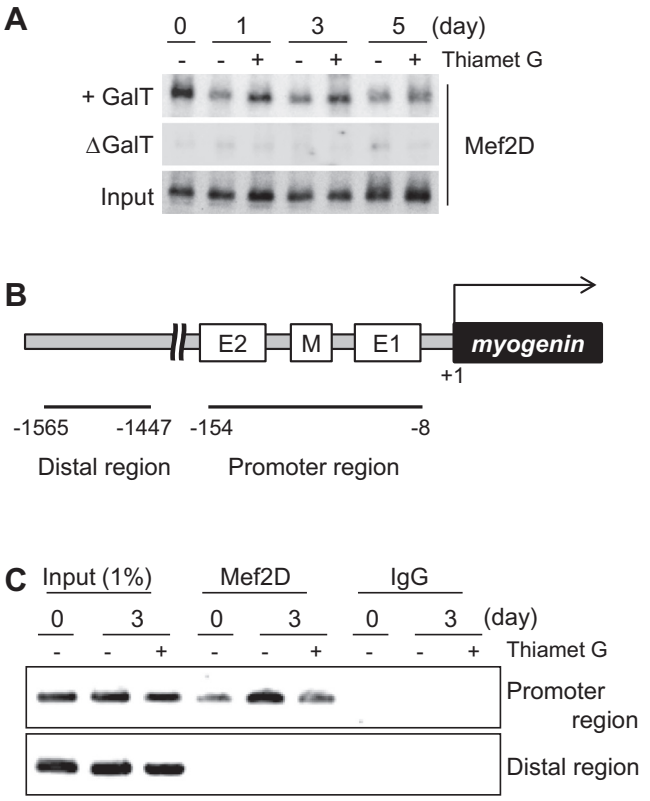

Fig. 4. Decreased *O*-GlcNAc glycosylation of Mef2D is required for myogenesis-dependent its recruitment to *myogenin* promoter region. (A) C2C12 myoblasts were induced to differentiate in the absence or the presence of 50 nM of thiamet G for 5 days and cell lysates were prepared. *O*-GlcNAc glycosylated proteins were specifically biotinylated and purified by streptavidin-resin. Representative Western blots of the biotinylated proteins probed with anti-Mef2D antibody are shown (+GalT panel). ΔGalT was a negative control experiment of biotinylation. (B) Schematic diagram of the promoter region of mouse *myogenin* gene. The promoter region (−154 to −8), which includes both MEF2-binding site (M) and E-boxes (E1 and E2), and the distal region (−1565 to −1447) were amplified with specific primers, respectively, in panel (C). (C) C2C12 myoblasts were induced to differentiate in the absence or the presence of 50 nM of thiamet G for 3 days. Chromatin immunoprecipitation was performed with anti-Mef2D antibody (Mef2D) and representative images are shown. Normal mouse IgG (IgG) was used as a negative control.

Acknowledgment

We are grateful to Dr. Gerald W. Hart (Johns Hopkins University School of Medicine) for the kind gift of CTD110.6 anti-*O*-GlcNAc antibody.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.033.

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