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Activation and Function of Murine Cyclin T2A and Cyclin T2B During Skeletal Muscle Differentiation

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ABSTRACT

Cyclin-dependent kinase 9 (Cdk9) is a serine-threonine kinase, involved in many cellular processes. The regulatory units of Cdk9 are the T family Cyclins (T1, T2) and Cyclin K. Cyclin T2 has two forms termed Cyclin T2a and Cyclin T2b that arise by an alternative splicing of the primary transcript. Upon induction of muscle differentiation, MyoD recruits Cdk9/Cyclin T2 on muscle-specific gene promoter sequences. This complex is able to phosphorylate the C-terminal domain of RNA polymerase II, enhancing MyoD function and promoting myogenic differentiation. This work focuses on the characterization of two murine Cyclin T2 isoforms and the evaluation of the role of Cdk9/Cyclin T2 complexes during the skeletal muscle differentiation. This study demonstrated a predominant expression of isoform b in all stages of differentiation. Moreover, both isoforms of Cyclin T2 are able to activate the myogenic program but Cyclin T2b has a predominant role, in particular during the latest stages. J. Cell. Biochem. 114: 728–734, 2013.

KEY WORDS: CYCLIN T2; CDK9; MYoD; SKELETAL MUSCLE DIFFERENTIATION; RNA POLYMERASE II PHOSPHORYLATION

Proliferation, terminal differentiation, and identity of skeletal muscle cells are controlled by the activity of several transcription factors.

Specifically, an important "modulatory" role in the development of skeletal muscle tissue is performed by the bHLH proteins, a family of myogenic transcription factors, which shares a basic helix-loop-helix DNA-binding domain. This transcription factors family includes MyoD, myogenin, Myf-5, and MRF4 or Myf-6/herculin, factors [Olson, 1992]. bHLH proteins are able to bind DNA-specific sites, called E box (CANNTG), located in enhancer and promoter sequences of muscle-specific genes [Lassar et al., 1989], and to induce their activation [Lassar and Münsterberg, 1994; Molkentin and Olson, 1996; Yun and Wold, 1996; Arnold and Winter, 1998].

MyoD has a crucial role in skeletal myogenesis by regulating more than 300 genes [Bergstrom et al., 2002]. Each MyoD monomer forms two α-helices interrupted by a short stretch of amino acids modeled as a loop. The basic domain fits in the major groove of the DNA, establishes most of the DNA–protein interactions and it is involved in the activation of transcription [Davis and Weintraub, 1992; Puri and Sartorelli, 2000]. Instead, two α-helices organize the dimerization interface for the formation of heterodimers.

In muscle differentiation, one of the co-activators of MyoD required for the completion of the myogenic program is the complex pTEFb [Simone et al., 2002a]. This complex is involved in the promotion of transcription elongation via phosphorylation of the carboxyl-terminal domain (CTD) of RNA polymerase II (RNA Pol II) and in the conversion of the inactive unphosphorylated, pre-initiation complex into the phosphorylated and active form. The essential residues in transcription elongation are serine 2 and 5 [Marshall et al., 1996; Simone et al., 2002a,b; Soutoglou, 2002;

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Marchesi et al., 2012]. The main subunits of pTEFb complex is Cyclin-dependent kinase 9 (Cdk9), previously named PITALRE [Graña et al., 1994], a cdc2-related serine/threonine kinase, widely expressed in human and murine tissues with high protein levels in terminally differentiated cells [de Luca et al., 1997; Bagella et al., 1998, 2000; Simone et al., 2002a,b]. There are two isoforms of Cdk9 in mammalian cells, $Cdk9_{42}$ and $Cdk9_{55}$ (in reference to their molecular weights) otherwise distributed in various tissues [Shore et al., 2003, 2005; Liu and Herrmann, 2005; Giacinti et al., 2008].

Cdk9 regulation and activity strictly differ from other CDKs because it is not cell cycle-dependent and it does not appear to be required in cell cycle progression [de Falco Giordano, 1998]. Cdk9 is regulated by Cyclins T (T1, T2) and cyclin K [Fu et al., 1999]. Cyclin T1 and T2 (CycT1, CycT2) share a highly conserved amino terminal motif (cyclin box region, 81% identity in human T-cyclins), a putative coiled-coil motif, a His-rich motif (responsible of the protein–protein interactions with the CTD of RNA Pol II) and a carboxy-terminal PEST sequence (less conserved than cyclin box region, 46% identity in human T-cyclins). The cyclin homology box, formed by 290 amino acids, is the most conserved region among different members of the cyclin-family and it is responsible for binding with Cdk9 [Peng et al., 1998; de Luca et al., 2003]. Interestingly, CycT2 bears a leucine-rich stretch next to its cyclin box capable to bind to CTD of RNA Pol II, thus providing an extra domain capable of targeting RNA Pol II [Peng et al., 1998; Kurosu et al., 2004].

CycT2 has two isoforms, T2a and T2b, (CycT2a, CycT2b) that likely arise by an alternative splicing of the primary transcript, which share the first 642 amino acids but have different carboxyl termini [de Luca et al., 2003].

All pTEFb complexes are able to phosphorylate components of the negative transcription elongation factor, DRB sensitivity-inducing factor (DSIF), and the negative elongation factor (NELF). Cdk9-dependent post-translational modifications are essential to reduce transcriptional repression, for the activation of splicing and polyadenylation machineries allowing a productive transcriptional elongation [Peterlin and Price, 2006]. Nevertheless, CycT1 or CycT2 are not redundant, and these different pTEFb complexes regulate subsets of distinct genes that are important for embryonic development [Kohoutek et al., 2009; Ramakrishnan et al., 2011].

In muscle differentiation, it has been demonstrated that Cdk9 directly interacts with MyoD in vitro [Simone et al., 2002a]. Moreover, it has been shown that Cdk9, in muscle cells, takes part to a multimeric complex containing MyoD, CycT2, p300, PCAF, and Brg1 [Giacinti et al., 2005]. This complex binds muscle-specific gene promoter regions and promote gene expression by inducing chromatin remodeling, through acetylation of specific lysine residues of histones H3 and H4 and phosphorylation Cdk9-dependent of RNA polymerase II CTD [Simone et al., 2004; Giacinti et al., 2005; Simone and Giordano, 2006; Giacinti et al., 2008]. Significantly, CycT1 was not detected in the same regions, suggesting a CycT2-depedent Cdk9 activation in skeletal myogenesis [Giacinti et al., 2005].

The elucidation of mechanisms governing muscle-specific transcription will provide important insight to better understand the embryonic development of muscle at the molecular level. The purpose of this study is the characterization of two murine CycT2 isoforms, CycT2a and CycT2b and the evaluation of their role in muscle differentiation program.

MATERIALS AND METHODS

CLONING AND SEQUENCING

Rapid amplification of cDNA ends (RACE) was employed to generate complete cDNA sequence encoding CycT2 isoforms. Mouse skeletal muscle Marathon-ready cDNAs (Clontech) were used as templates in RACE polymerase chain reaction (PCR) to obtain the 5′ and 3′-end cDNA fragment according with the manufacture's protocol. Specific primers for CycT2 murine isoforms were originated by a partial murine CycT2 cDNA obtained by a previous screening of a Lambda ZAP Express cDNA Library derived from a mouse embryo at 14.5 days post-coitum (Stratagene, La Jolla, CA) using human CycT2 cDNA fragment as a probe (data not shown).

The PCR for 5′-end was carried out using Adaptor primer 1, included in the kit, as sense primer and CycT2-reverse (GCTTGCAAATGGTCCAATTGGG) as antisense primer.

The PCR for 3′-ends was carried out using CycT2a-forward and CycT2b-forward (CCACGGTGCTCAGGAGTCCT; CAGCGGATGGAATGCCTCCC, respectively) as sense primers and Adaptor primer 1 as antisense primer.

PCR products were cloned into pGEM-T Easy Vector System II (Promega) and then sequenced. Sequences were used to design the primers for full-length amplification:

CycT2.for: GGATCCATGGCGTCGGGCCGTGGA;
CycT2a.rev: GGATCCCTGGAGTCAGGACCGTGGGGCTCC;
CycT2b.rev: GGATCCTTACATATTCATTCCTTG

PLASMIDS

Plasmids myogenin-luciferase myosin heavy chain (Myh)-luciferase promoter were constructed by PCR. Genomic DNA was extracted by DNeasy blood and tissue kit (Qiagen) following the manufacturer's protocol. PCR were performed with specific primers:

Myogenin-promoter.for: CAAACGCTAGCCAGCTCTCACGGCTGCTATGA
Myogenin-promoter.rev: GGGAGATCTGGTAGAAATAGGGGGATGTCTC
Myh-promoter.for: CTCCCGGGCTGTATTTCCTCATCTGTGAGGA
Myh-promoter.rev: CTACAAGCTTAGACCAGTTGCTCCTATGCCC

Amplified products were cloned in the *Nhe*I–*Bgl*II and *Xma*CI–*Hin*dIII sites of the pGL3 basic vector (Promega) for Myogenin promoter and Myh promoter, respectively.

Plasmids pcDNA3-CycT2a and pcDNA3-CycT2b were constructed by inserting the murine full-length cDNAs into vector pcDNA3 (Invitrogen).

The correct sequences of all constructs were confirmed by sequencing. Constructs pcDNA3-cdk9wt and pcDNA3-MyoD

expressing full-length have been previously described [de Falco et al., 2000; Simone et al., 2002a].

CELL CULTURE AND DIFFERENTIATION

Myoblasts (C2C12, ATCC) were grown in DMEM supplemented with 20% FBS 1% L-glutamine and antibiotics (Growth Medium, GM). Myogenesis was induced by serum withdrawal in the presence of 2% horse serum (differentiation medium, DM). Pellets were collected every 24 h for 144 h.

IMMUNOBLOTTING

Cells were lysated in lysis buffer (20 mM Tris–HCl pH 8; 137 mM NaCl; 10% glycerol 1% Nonidet P-40; 2 mM EDTA; Protease Inhibitor Cocktails).

Protein extract (50 μg) was resolved in 8–10% SDS/PAA gel and transferred to a nitrocellulose membrane. Protein levels were detected with the followed antibodies anti-Cdk9 (Rockland), Anti-CycT2, anti-MyoD anti-Myogenin, anti-Myh (Santa Cruz). Equal loading was controlled with anti-GAPDH and anti-Hsp70 (Santa Cruz).

Anti-mouse, rabbit (1:10,000), goat (1:2,500) peroxidase conjugated (Pierce) and ECL detection system (PerkinElmer) were used for detection.

TOTAL RNA EXTRACTION, cDNA SYNTHESIS AND REAL-TIME PCR

Total RNA was extracted from 5–10 $\times$ 10^6 cells using High Pure RNA Isolation Kit (Roche). One microgram of RNA was used for cDNA production with random primers, using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), following the manufacturer's protocol. Real-time was performed using 250 nM primers and FastStart Universal SYBR Green Master (ROX), (Roche). Amplification conditions for GAPDH, CycT2, MyoD, Myogenin, Myh amplicons were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 30 s. Amplification conditions for Cdk955 amplicon were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 72°C for 1 min.

Accumulation of fluorescent products was monitored using Applied Biosystem 7300 system. Each data point was obtained from at least three independent experiments. Transcripts for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as a reference. To ensure specific PCR amplification, every real-time PCR run was followed by a dissociation phase analysis (denaturation curve) and by gel electrophoresis. $\Delta\Delta C_t$ method was used to calculate relative changes in gene expression; primer efficiency was calculated for every target using 5$\times$ 10-fold serial dilutions of PCR products. Specific primer sequences are reported below:

GAPDH: for AGAAGGTGGTGAAGCAGGCATC; rev CGAAGGTGGAAGAGTGGGAGTTG

CycT2a: for CGTCTCCTCCGCCTCCAGTG; rev AGATGTCCGTAGCCCACCTGC

CycT2b: for AGCGAAGCCTCCCACAACC; rev GTCCGTAGCCCACCTGGTATG

Cdk9: for TCCATCACCCCAGAGGTGTG; rev CAGCTTGTCAATGAGGTCCAG

Cdk955: for GGAGCCTCCGCCCCCAGGCCTTCC; rev CGGCGGTGCGTCCCGCTGCATTG

MyoD: for GATGGCATGATGGATTACAGCG; rev GGAGATGCGCTCCACTATGCT

Myogenin: for CAATGCACTGGAGTTCGGTCC; rev AGTGATGGCTTTTGACACCAAC

Myh: for CAGAGCTTATTGAGATGCTTCTG; rev ATCACAGCGCCTGTGAGCTTG

TRANSIENT TRANSFECTIONS AND LUCIFERASE ASSAY

Transient transfections were performed using FuGene HD (Roche applied). Two micrograms of total DNA diluted in 100 μl of Opti-MEM (CellGro) was incubated with 8 μl of FUGeneHD for 20 min to let the transfection complex form. The transfection complex was added in a ratio 1:16 to the volume of the cell incubation medium (6 μl of transfection complex was added to 100 μl of complete medium in 96-well plate).

Dual luciferase reporter assay (Promega) was used to measure the firefly luciferase and Renilla luciferase activity within the transfected cells. Each experiment was conducted as suggested by the manufacturer. Luciferase assay was conducted on C2C12 myoblasts transfected with Myogenin-luc reporter, Myh-luc reporter, RL-TK Renilla, and expression vectors for CycT2a, CycT2b, Cdk9, and MyoD. Transfected cells were cultured in DM for 24 and 48 h. Luciferase activity was normalized to TK-directed Renilla expression, in a ratio 1:20 respect to firefly luciferase vector, to control for variability in transfection efficiency. The assay was performed with Sirius Luminometer (Berthold detection systems).

Results are expressed in arbitrary units relative to the activity of the basic luciferase vector (pGL3-myogenin/Myh promoter).

IMMUNOFLUORESCENCE

Cells were fixed with 4% paraformaldehyde in PBS for 15 min at RT and permeabilized with 0.1% TritonX-100 (Fisher Scientific, Pittsburgh, PA) in PBS for 10 min at room temperature. Nonspecific binding sites were blocked with PBS containing 6% bovine serum albumin (BSA; Sigma–Aldrich) for 1 h at RT. Cells were then incubated overnight at 4°C with anti-CycT2a and anti-CycT2b antibodies (Santa Cruz Biotechnology), in PBS with 1% BSA. Secondary antibody, Alexa Fluor 594 rabbit anti-goat IgG (Invitrogen) was diluted 1:600 in PBS containing 1% BSA. Nuclear staining was performed with 100 ng/ml of DAPI (4′,6′-diamino-2-phenylindole; Molecular Probes), 1 min at RT. After washing, glasses were mounted with Vectashield mounting medium (Vector Laboratories) and visualized with Olympus IX81 fluorescence microscope (Olympus Microscopes).

RESULTS

ISOLATION AND CHARACTERIZATION OF THE MURINE CycT2 cDNA

In order to characterize murine isoforms of CycT2, full-length coding regions of two proteins were amplified from mouse skeletal muscle Marathon-Ready cDNA by PCR. Purified PCR products were cloned into pGEM-T Easy Vector System II and sequenced. Sequences of mouse CycT2a and CycT2b were compared with human isoforms. Murine transcripts showed 86% and 87% of

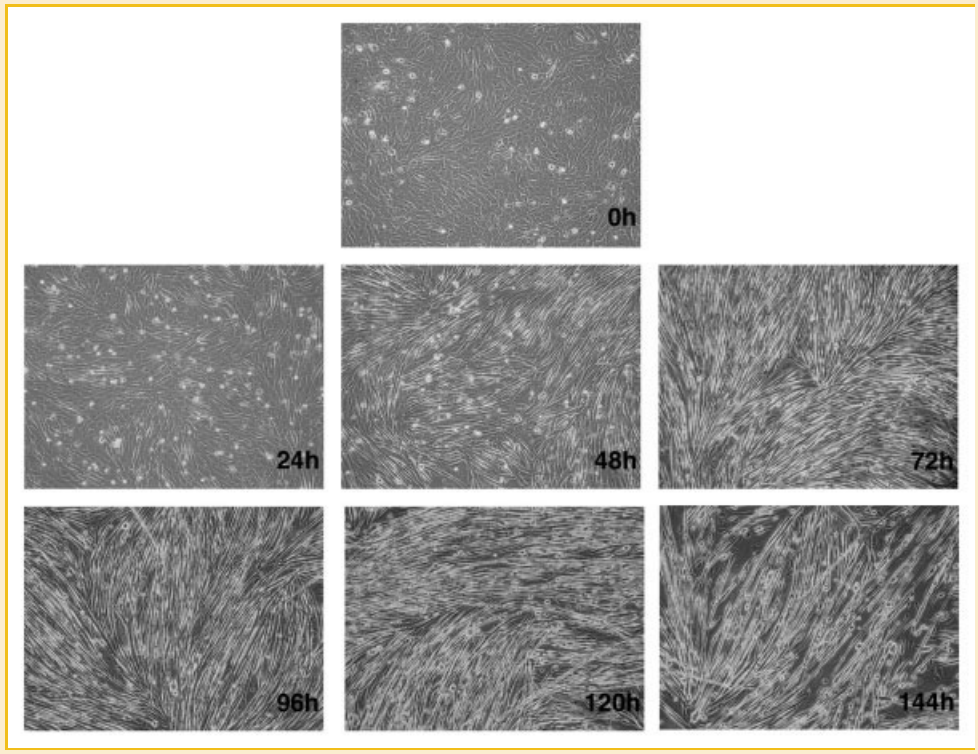

Fig. 1. C2C12 differentiation. Myoblasts (C2C12) were grown to 90% confluence in complete medium (GM, 20% FBS) and induced to differentiate by serum withdrawal in the presence of 2% horse serum (DM) for 144 h. Pellets were collected every 24 h.

similarity respect to human counterparts. Instead, the comparison between the amino acidic sequences showed 85% and 86% of similarity (Supplementary Figs. S1 and S2). Interestingly, the amino acid lengths of two murine isoforms, CycT2a and CycT2b, were shorter of six and seven units compared to the human counterparts, respectively. Predicted molecular weights of CycT2a and CycT2b are 73 and 80 kDa, respectively. The sequences of CycT2a and CycT2b have been deposited in the GeneBank database (accession number JX965957 and JX965958 for CycT2a and CycT2b respectively).

MURINE MYOBLASTS EXPRESS HIGHER LEVELS OF CycT2b RESPECT TO CycT2a IN ALL STAGES OF SKELETAL DIFFERENTIATION

Several studies identified Cdk9-CycT2 as essential complexes and co-activators during the myogenic program. Assays carried in mouse embryonic fibroblast cell lines demonstrated the role of isoform a of CycT2 [Simone et al., 2002a] but a functional role of isoform b has not yet been demonstrated.

In order to analyze the expression of CycT2 isoforms during skeletal muscle differentiation, the process was induced in murine myoblasts (C2C12) through serum-deprivation in presence of horse serum, for 144 h. Pellets were collected each 24 h. The process was monitored evaluating cell phenotype, C2C12 stop proliferating and complete myotube formation approximately 72 h after induction (Fig. 1).

CycT2a, CycT2b, and Cdk9 expression levels were analyzed by immunoblotting and real-time PCR in myoblasts under proliferative conditions and at several stages of differentiation. Moreover, MyoD,

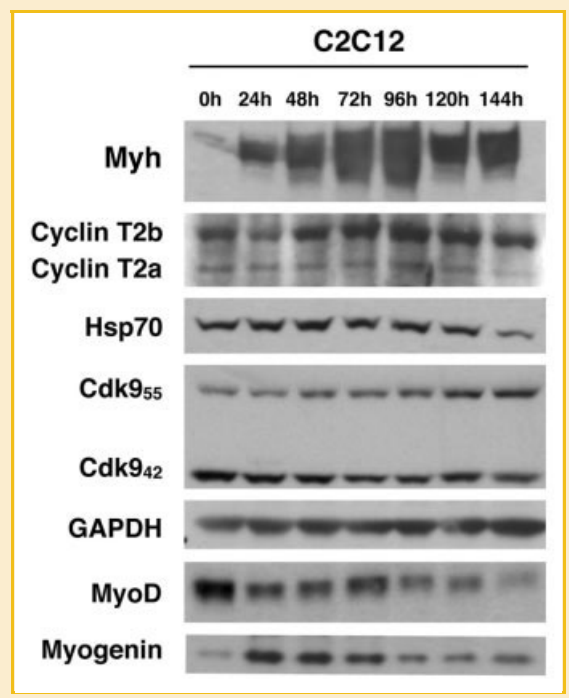

Fig. 2. Protein expression during skeletal muscle differentiation. Immunoblot analysis was performed with the followed antibody Anti-Cyclin T2, anti-Cdk9, anti-MyoD anti-Myogenin, anti-Myh. Equal loading was controlled with anti-Gapdh and anti-Hsp70.

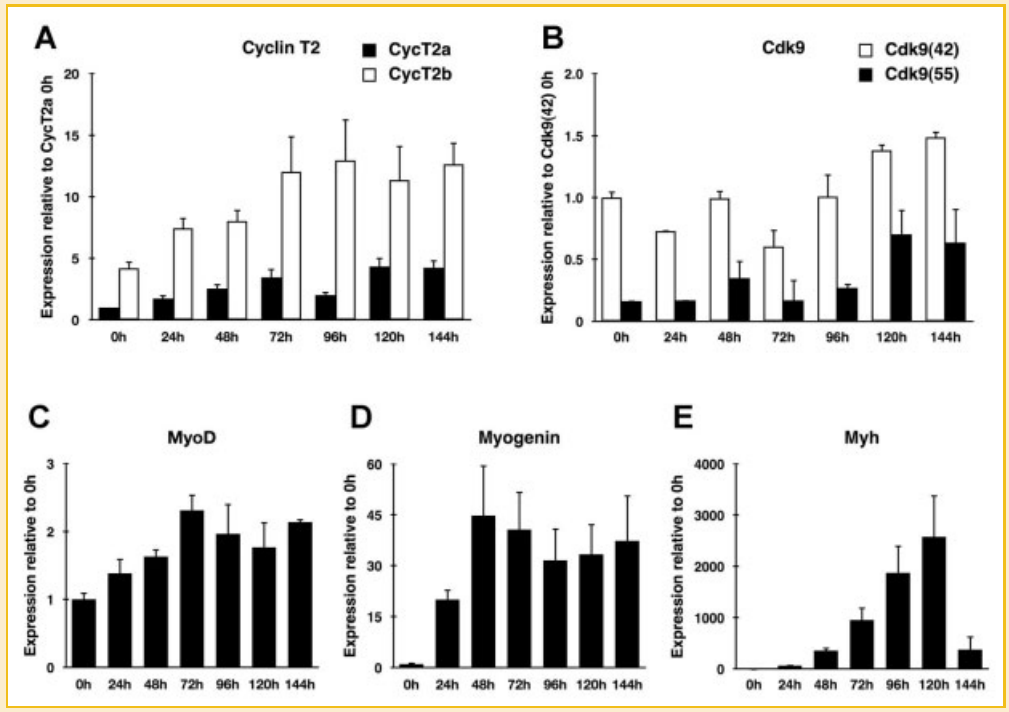

Fig. 3. Relative mRNA expression during skeletal muscle differentiation. qPCR was performed using cDNA from myoblasts at several stage of differentiation. In order to distinguish two Cyclin T2 (A) and Cdk9 (B) isoforms, specific primers were designed in the splicing site region of Cyclin T2 and in 5′ sequence of Cdk9. Myogenesis was verified by determination of mRNA expression of MyoD (C), Myogenin (D) and Myh (E). The reported data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

myogenin, and Myh levels were also examined in order to verify the correct expression of muscle-specific genes (Figs. 2 and 3C–E).

Immunoblot showed that CycT2b levels, in comparison to CycT2a were markedly higher in all phases of skeletal muscle differentiation, particularly in later stages. In contrast, levels of Cdk9 did not change during the process except for 55 kDa isoform, which slightly increased in the advanced stages (Fig. 2).

These results were confirmed by transcript analysis (Fig. 3). Unlike Cdk9 isoforms, which showed a constant expression with a slight increase only in latest stages of differentiation (Fig. 3B) CycT2 expression increased during the process, especially the isoform b. Indeed, differences on CycT2 isoforms expression were between 2.5 (proliferating myoblast) and 7 (144 h after myogenesis induction; Fig. 3A).

In order to identify differences in CycT2 subcellular localization, immunofluorescence assays was conducted in C2C12 cells in proliferative and differentiation conditions.

As showed in Figure 4, incubation with anti-CycT2a and anti-CycT2b demonstrated perinuclear localization in myoblasts cultured in GM (0 h) and a nuclear localization in differentiating cells (24 and 72 h). Differences between CycT2a and b in subcellular localization were not detected.

Cdk9/CycT2 COMPLEXES ENHANCES THE TRANSCRIPTIONAL ACTIVITY OF MYoD, CycT2b HAVE A PREDOMINANT ROLE IN THE LATEST STAGES OF THE MYOGENESIS

In order to establish functional differences between CycT2a and CycT2b, we compared effects of CycT2 isoforms overexpression on MyoD-dependent transcription.

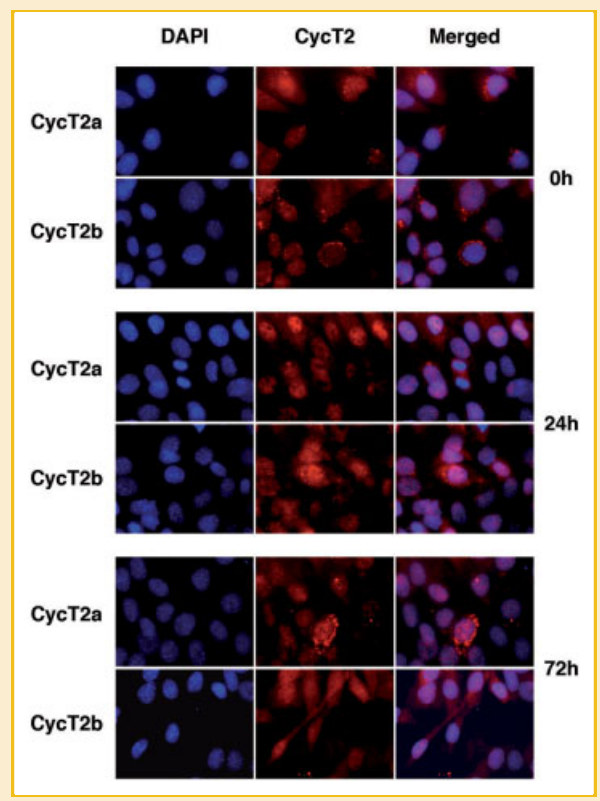

Fig. 4. Subcellular localization of Cyclins T2 during skeletal muscle differentiation. C2C12 in proliferation and induced to differentiate for 24 and 72 h, are stained with anti-cyclin T2a and anti-cyclin T2b. Nuclei are labeled with DAPI.

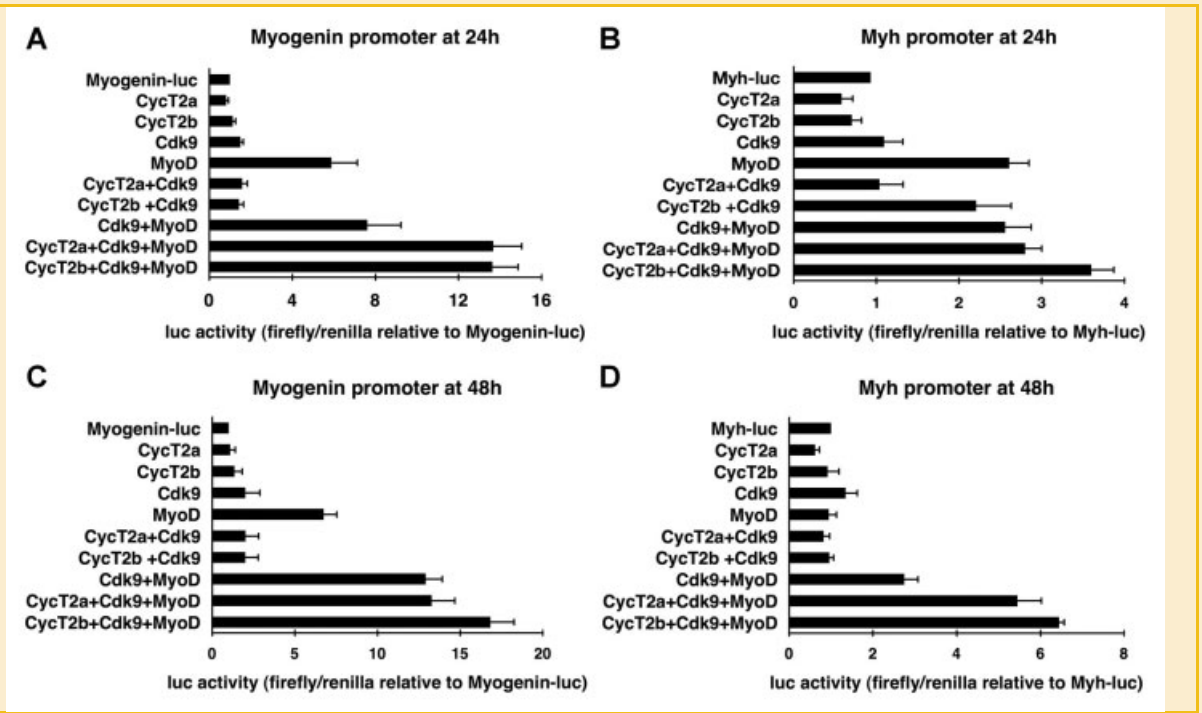

Fig. 5. Regulation of Myogenin promoters by Cyclin T2. Luciferase assay was conducted on C2C12 myoblast transfected with Myogenin-luc (A,C), Myh-Luc (B,D) reporter vectors, RL-TK Renilla and expression vectors for CycT2a, CycT2b, Cdk9, and MyoD. Transfected cells were cultured in DM for 24 h (A,B) and 48 h (C,D). Luciferase activity was normalized to TK-directed Renilla expression. Results are expressed in arbitrary units relative to the activity of the basic luciferase vector (Myogenin-luc, Myh-luc).

Myogenin-luciferase reporter (Myogenin-luc) and Myh-luciferase reporter (Myh-luc) were transiently transfected into C2C12 cells, and the influence of Cdk9 and CycT2 alone, or in combination, on the transactivation potential of MyoD, was assayed after 24 or 48 h in differentiation medium.

Transfection of either individual or pairwise combinations of Cdk9, CycT2a, and CycT2b expression vectors had no effect on Myogenin-luc and Myh-luc in the absence of MyoD. Conversely, MyoD-dependent transactivation of promoters was increased, by co-expression of both complexes Cdk9/CycT2 (Fig. 5). At 24 h, CycT2a and CycT2b had the same effect on myogenin-luc reporter (Fig. 5A); at 48 h the effect of CycT2b in comparison to the effect of CycT2a was stronger (Fig. 5C). On Myh-luc reporter the effect of CycT2b was stronger at both 24 h and 48 h (Fig. 5B,D). These results suggest a predominant role of CycT2b in latest stages of differentiation process.

DISCUSSION

During cell division, cyclins play an essential role being subjected to cyclical expression and ubiquitin-dependent degradation, and acting as regulatory subunits of complexes with the cyclin-dependent kinases (Cdks) [Sherr, 1996]. Some Cdks/Cyclin, as Cdk7/Cyclin H, Cdk8/cyclin C, and Cdk9/Cyclin T, seem to direct their activity in a cell cycle independent manner and appear to be involved in other processes as signal transduction, apoptosis, differentiation, and transcription during the initiation or the elongation steps [Dynlacht, 1997; de Luca et al., 2003].

A previous study showed that Cdk9, in association with Cyclin T2, plays an important role in the activation of the myogenic program [Simone et al., 2002a]. Upon induction of muscle differentiation, MyoD recruits Cdk9/CycT2 on muscle-specific gene promoter sequences. This complex is able to phosphorylate the (CTD) of RNA PolII, enhancing MyoD function and promoting myogenic differentiation [Giacinti et al., 2005].

This work focuses on the evaluation of Cdk9/CycT2 complexes' role during the skeletal muscle differentiation. In order to understand the role of CycT2 isoforms in muscle differentiation, myogenesis was induced in murine myoblast and mRNA and protein levels were analyzed at several stage of differentiation process. In addition, luciferase assay performed allowed to identify functional differences between two complexes.

Results demonstrated that CycT2b levels, both mRNA and protein expressions, in comparison to CycT2a are markedly higher in all the stages of differentiation. These results assumed a major role for CycT2b in muscle differentiation process. Moreover, the luciferase assay showed that both CycT2 isoforms were able to increased MyoD-dependent transactivation of Myogenin and Myh promoters. During the first stages of differentiation both CycT2 activated the muscle differentiation program but during the latest stages the activity of CycT2b was stronger.

The higher expression and differences of functional activity showed by CycT2b suggest a predominant role of this protein in the differentiation process, in particular during the latest stages. These studies highlight a critical role for Cdk9/CycT2b complex in controlling skeletal muscle growth and differentiation. Our observations point

toward the cooperation with MRFs, such as MyoD and Myogenin, suggesting that CycT2b has a crucial role in maintaining cells at the differentiation terminal stage. The understanding of the functional properties of the Cdk9/CycT2 complexes and the interaction with specific associated partners will help us to clarify the complex mechanisms that regulate the myogenic program.

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