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Pctaire1/Cdk16 promotes skeletal myogenesis by inducing myoblast migration and fusion

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ABSTRACT

The Cdk-related protein kinase Pctaire1/Cdk16 is abundantly expressed in brain, testis and skeletal muscle. Functional roles of Pctaire1 such as regulation of neuron migration and neurite outgrowth thus far have been mainly elucidated in the field of nervous system development. Although these regulations based on cytoskeletal rearrangements evoke a possible role of Pctaire1 in the development of skeletal muscle, little is known in this regard. In this study, we demonstrated that myogenic differentiation and subsequent fusion is promoted in Pctaire1 overexpressing cells, and conversely, is inhibited in the knockdown cells. Furthermore, our findings suggest that Pctaire1 exerts promyogenic effects by regulating myoblast migration and process formation during skeletal myogenesis.

1. Introduction

Myogenic differentiation is an essential process for muscle growth and homeostasis during embryonic development and in postnatal life. Myogenesis is accomplished by undergoing multistep process accompanied by a complex series of molecular and morphological changes. After proliferation, followed by cell cycle withdrawal, mononucleated myoblasts differentiate into elongated myocytes, then migrate, adhere and fuse to one another to form multinucleated myotubes, and mature muscle fiber in the end [1–3]. The myogenic program is orchestrated by sequential activation of myogenic regulatory factors (MRFs), the basic helix–loop–helix (bHLH) family of transcription factors (Myf5, MyoD, myogenin and MRF4), which can induce expression of muscle-specific genes in concert with transcriptional cofactors such as Mef2 and E proteins [4–9].

Accumulating evidence suggests that protein kinases functioning as substrates for caspase-3 play a key role in regulating differentiation [10–13]. As previously reported, we have identified 30 protein kinases as novel substrates for caspase-3 [14], and investigated their function in skeletal muscle differentiation [13]. In consequence, we could pick out Pctaire1 kinase as one of the positive regulators of myogenic differentiation as in the case of previous report regarding Nek5 [13].

A serine/threonine kinase Pctaire1 (now known as Cdk16) is one of the atypical members in cyclin-dependent kinase (Cdk) family, and has both N- and C-terminal extensions that are divergent from or absent in other Cdks. These additional domains play important roles in mediating protein–protein interactions and were shown to regulate kinase activity [15–17]. Activity of Cdks is generally regulated by binding to an activating subunit, cyclin. Unlike conventional cyclin-Cdk interactions, however, not only the catalytic domain but also domains within the N-terminal extension of Pctaire1 are required for binding to its activator cyclin Y [18]. Although Pctaire1 activity has been shown as cell cycle regulated, it remains uncertain whether Pctaire1 itself is involved in cell cycle progression in the same way as some other Cdks [17,19]. Also, an alternative regulatory mechanism has been proposed to activate and inhibit the kinase activity of Pctaire1 through phosphorylation by Cdk5/p35 and protein kinase A (PKA), respectively [16,20].

Pctaire1 is ubiquitously expressed in mammalian tissues including skeletal muscle and is particularly abundant in terminally differentiated cells in brain and testis, and in transformed cell

Abbreviations: DM, differentiation medium; GM, growth medium; MHC, myosin heavy chain; MRFs, myogenic regulatory factors

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lines [16,20–22]. During development of mouse brain, Pctaire1 cooperates with Cdk5 to initiate a kinase cascade that governs cytoskeletal rearrangements essential for neuron migration and neurite outgrowth by the myocardin-related transcription factors (MRTFs)/serum response factor (Srf) pathway [23]. In human cell lines, Pctaire1 is phosphorylated at several residues, including Ser119 and Ser153, which have been shown to be substrates for PKA in vitro [16,17]. Phosphorylation of S119 and S153 creates 14-3-3 consensus binding motifs, and alanine mutation of both residues abolishes the binding of Pctaire1 to 14-3-3 in the presence of active PKA, which also results in the inhibition of neurite outgrowth in Neuro-2A cells [16]. However, the precise role of this modification and 14-3-3 binding remains to be elucidated. In addition to the function in the nervous system, by generating a conditional Pctaire1-knockout mouse, Mikolcevic et al. found that Pctaire1 is essential for the completion of spermatogenesis [17]. Moreover, Pctaire1 has also been reported to be involved in the regulation of intracellular vesicles and secretion [24,25].

In this study, we focus on the process of myogenesis in which the role of Pctaire1 remains to be defined though its functions involved with cytoskeletal rearrangements and exocytosis in nervous system are linked to the process of muscle cell fusion [26]. We report here, Pctaire1 is involved in the promotion of myogenic differentiation, and fusion by inducing migration and process formation of myoblasts.

2. Materials and methods

2.1. Antibodies

Antibodies against the following antigens were used: myosin heavy chain (MHC) (clone MF20: anti-all isoforms, Developmental Studies Hybridoma Bank, Iowa City, IA), troponin C (sc-20642, Santa Cruz Biotechnology, Santa Cruz, CA), Pctaire1 (#4852, Cell Signaling Technology, Beverly, MA), V5 epitope (Invitrogen, Carlsbad, CA), cleaved caspase-3 (#9664, clone 5A1E, Cell Signaling Technology) and α-tubulin (clone DM1A, Sigma, St. Louis, MO).

2.2. Plasmid constructs

To construct expression plasmids for mammalian cells, cDNAs encoding human MyoD1 (RefSeq: NM_002478), human PCTAIRE1 (RefSeq: NM_006201) and mouse Pctaire1 (RefSeq: NM_011049) were subcloned into vectors (pcDNA3.1/nV5-DEST, pcDNA3.2/V5-DEST, and pcDNA6.2/N-EmGFP-DEST) (Invitrogen). Mutants of human PCTAIRE1 were generated by site-directed mutagenesis using a PrimeSTAR mutagenesis basal kit (Takara Bio Inc., Otsu, Japan).

2.3. Cell culture and transfection

C2/4 [27] is a subclone of C2C12 mouse myoblast cell line, and is referred to simply as C2C12 herein. C2C12 cells were maintained at 37 °C in a 5% CO_2 incubator in a growth medium (GM) of Dulbecco's modified eagle medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), 2 mM L-glutamine (GIBCO, Grand Island, NY), and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) (GIBCO), as previously reported [13]. Myogenic differentiation of C2C12 myoblasts was induced by replacing the GM with a differentiation medium (DM) that is DMEM with high glucose (Wako, Osaka, Japan) supplemented with 2% horse serum and penicillin–streptomycin. Unless otherwise noted, cells were cultured in GM, and all transfections in this study were performed in a semi-confluent condition using TransIT-LT1 transfection reagent (Mirus, Madison, WI) according to the manufacturer's instruction.

2.4. Western blot analysis

All Western blot analyses were performed as described previously [28]. Briefly, proteins in whole-cell lysates were separated by SDS–PAGE and transferred onto a PVDF membrane by electroblotting. After blocking with 5% milk/TBST, the membrane was probed with a given primary antibody and then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody. The immunoreactive proteins were visualized using Immobilon Western HRP substrate Luminol Reagent (Millipore, Bedford, MA) and LAS-4000 mini biomolecular imager (GE Healthcare, Piscataway, NJ).

2.5. Immunostaining analysis

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature, and then permeabilized with 0.5% Triton X-100 in PBS for 5 min. After blocking with 5% calf serum in TBST for 1 h, the cells were incubated with anti-troponin C antibody, anti-MHC antibody or anti-V5 antibody for 1 h at 37 °C. The cells were then incubated with Alexa Fluor 488- and/or 568-conjugated appropriate secondary antibodies (Molecular Probes, Eugene, OR) for 1 h at room temperature. DAPI staining was carried out concomitantly with the above procedure. Fluorescence images were acquired with the Carl Zeiss LSM710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

2.6. Real-time quantitative RT-PCR analysis

Total cellular RNA was extracted from C2C12 cells using a QIAeasy RNA mini kit (Qiagen, Valencia, CA). Extracted total RNA was subjected to reverse transcription with a Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Schweiz). Real-time qRT-PCR analysis was performed using a LightCycler 480 Real-time PCR System (Roche). The following primer sets were designed by the software of Assay Design Center (Roche): 5′-CGCAATCGG ATCTCTGCT-3′ (forward) and 5′-CCTCCTTTAGTGCAAATATGGAA-3′ (reverse) for Pctaire1; 5′-TGAAGATAGTGATTTTATTTTCAAACG-3′ (forward) and 5′-GACAGACATTGAGAAGTTCTGAGG-3′ (reverse) for Mef2a; and 5′-TCTGCCCTCAGTCAGTTGG-3′ (forward) and 5′-CGTGGTGTGTTGTGGGTATC-3′ (reverse) for Mef2c. The values were normalized to GAPDH (primer set was purchased from Roche).

2.7. Lentivirus-mediated gene transfer and generation of stable cell lines

Lentivirus-mediated RNA interference (RNAi) was utilized for the generation of stable cell lines in which the expression of Pctaire1 is suppressed, and followed by knockdown analysis. For this purpose, an HIV-based self-inactivating lentiviral expression vector (RIKEN BioResource Center, Japan [29]) was slightly modified by adding a transgene cassette to drive the expression of a short hairpin RNA (shRNA), and the resulting expression vector was termed as CSII-CMV-GFP-IRES-Bsd-H1 [30]. In this study, the following sequences were selected as RNAi targets: 5′-GGTAGTTG CTGGTGAACAGAA-3′ (shPctaire1-#1) and 5′-GGTTTCCACACTAAGG CTAGG-3′ (shPctaire1-#2), corresponding to the nucleotide sequences in the 3′-UTR of mouse Pctaire1. Negative control sequences were 5′-CAACAAGATGAAGAGCACCAA-3′ (non-target shRNA control, i.e. shNC) and 5′-GACTACACAAATCAGCGAT-3′ (shLacZ). After construction of the shRNA expression plasmids by inserting the respective double-stranded oligonucleotides just downstream of the H1 promoter, lentiviral particles were generated according to standard transfection protocol. After