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Ubiquitin–proteasome-mediated degradation and synthesis of MyoD is modulated by αB-crystallin, a small heat shock protein, during muscle differentiation

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ABSTRACT

αB-crystallin, a small heat shock protein, plays an important role in muscle homeostasis. It gets up-regulated during muscle differentiation and mice lacking αB-crystallin die prematurely with extensive muscle wastage. We have examined the role of αB-crystallin in muscle development using C2C12 myoblasts as a model system. Over-expression of αB-crystallin delays the muscle differentiation program significantly. C2C12 myoblasts over-expressing αB-crystallin (CRYAB-C2C12) display defect in cell-cycle exit upon induction of differentiation. During differentiation, CRYAB-C2C12 cells exhibit sustained level of cyclin D1 and delay in p21 and myogenin expression as compared to C2C12 cells. We find less accumulation of MyoD in CRYAB-C2C12 cells than in C2C12 cells. *In vivo* protein stability studies reveal faster ubiquitin–proteasome-mediated MyoD degradation in CRYAB-C2C12 cells ($t_{1/2}$ = 1.42 h) than in C2C12 cells ($t_{1/2}$ = 2.37 h). Immuno-precipitation experiments showed that MyoD gets ubiquitinated at earlier time points in CRYAB-C2C12 cells than in C2C12 cells. Our data reveal alterations in the synthesis and degradation of MyoD in CRYAB-C2C12 cells. The level of αB-crystallin as well as its Ser-59 phosphorylated form increases with increasing time of differentiation. Our studies show, *inter alia*, that αB-crystallin modulates myogenesis by altering MyoD level and provide an interesting insight in its role in myogenesis.

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1. Introduction

Muscle differentiation involves proliferation of myoblasts, followed by cell-cycle arrest and terminal differentiation into multinucleated mature muscle fiber [1–3]. In the proliferating condition, myoblasts express significant amount of αB-crystallin (small heat shock protein), which gets induced upon differentiation [4,5]. αB-Crystallin acts as a molecular chaperone and prevents the stress-induced aggregation of target proteins [6–8]. Studies from our laboratory have shown that αB-crystallin undergoes structural alteration and displays enhanced chaperone activity at higher temperature [9,10]. We have shown that in heat-stressed C2C12 myoblasts, αB-crystallin migrates to the nucleus, where it colocalizes with lamins and SC-35 [11]. αB-crystallin seems to play an important role in muscle homeostasis. It gets up-regulated by 10-fold during muscle differentiation and mice lacking αB-crystallin die prematurely with extensive muscle wastage [5,12]. A point mutation in αB-crystallin, R120G, is known to be associated with desmin-related myopathy, where it forms aggregates with the desmin intermediate filaments [13,14]. All these findings clearly suggest the importance of αB-crystallin in muscle maintenance and differentiation.

Skeletal myogenesis is regulated by sequential and coordinated expression of a family of muscle-specific factors–Muscle Regulatory Factors (MRFs)–which include MyoD, Myf5, myogenin and MRF4 [15]. MyoD and Myf5 are expressed in proliferating undifferentiated myoblasts and are shown to be involved in the specification of myogenic lineage [16]. The decision to differentiate relies on the cross-talk between MyoD and cell-cycle signaling pathways. It is found that the over-expression of cyclin D1 results in an inhibition of MyoD-dependent transcription and decrease in p21 expression [17,18]. Another important parameter that governs myogenesis is the ability of myoblasts to survive during differentiating conditions. αB-crystallin was shown to protect myoblasts by preventing the activation of caspase-3 during differentiation [19]. Muscle differentiation has been shown to be inhibited during hypoxic condition through accelerated degradation of MyoD protein [20]. It is important to note that during hypoxic condition, αB-crystallin levels increase several fold and shown to exhibits protective function [21]. These reports clearly suggest an important role for αB-crystallin in myogenesis. However the molecular mechanisms of its involvement in muscle differentiation are not understood.

In the present study, we have investigated the role of αB-crystallin in muscle differentiation using C2C12 cells, mouse myoblasts as a model system. We find that differentiation is delayed significantly in C2C12 myoblasts stably transfected with αB-crystallin. We have probed the molecular details of this delay in differentiation: our studies show, *inter alia*, that αB-crystallin modulates myogenesis by altering cell cycle exit and MyoD levels.

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2. Materials and methods

2.1. Antibodies and reagents

DMEM, Fetal Calf Serum (FCS), DMSO, MTT and mouse monoclonal anti-Flag antibodies were purchased from Sigma Chemical Company, USA. Cycloheximide, MG132, rabbit polyclonal anti-Ubiquitin antibodies were obtained from Calbiochem, EMD Biosciences, Germany. Propidium Iodide (PI), Alexa-488- and Cy3-conjugated secondary antibodies were obtained from Molecular Probes, Invitrogen Corp. Oregon, USA. Anti-GAPDH mouse monoclonal antibodies were purchased from Chemicon International Inc., USA. Rabbit polyclonal antibodies for αB-crystallin, phosphoserine-59-αB-crystallin and phosphoserine-45-αB-crystallin and mouse monoclonal anti-cyclinD1 antibodies were obtained from Stressgen Biotechnologies, Victoria, Canada. Mouse monoclonal antibodies for MyoD were purchased from Dako Cytomation, USA; rabbit polyclonal anti-myogenin antibodies were from Santa Cruz, USA. Mouse monoclonal antibodies for p21 and phospho-p38 MAPK were from BD Transductions, Pharmingen, USA. Rabbit polyclonal cleaved caspase-3 (Asp175) antibodies were procured from Cell Signaling Technology, USA. EDTA-free protease inhibitor cocktail (PIC) containing bestatin, pefabloc SC, aprotinin, leupeptin, pepstatin, E-64, antipain-dihydrochloride was procured from Roche Applied Sciences, USA; Lipofectamine-2000 was obtained from Invitrogen, USA. HRPO-conjugated anti-rabbit, anti-mouse secondary antibodies and Enhanced Chemiluminescence (ECL) Western blot detection kit were purchased from Amersham Biosciences, USA. Vectashield mounting medium containing 4′, 6-diamidino-2-phenylindole (DAPI) was from Vector Laboratories, USA.

2.2. Plasmids and construction of FLAG-tagged cDNAs

cDNA of the full-length αB-crystallin was PCR-amplified using the following primers: Forward primer—5′-GGCCGAATTCATGGACATCGC-CATCCACCAC-3′and Reverse primer—5′-GCCCTCGAGCTATTTCTT-GGGGGCTGCGG-3′. The PCR products were digested with *Eco*RI and *Xho*I restriction enzyme and ligated into a modified pCDNA3 vector, in frame with the FLAG epitope, which was inserted upstream of the multiple cloning site. The final positive clones were confirmed by automated DNA sequencing.

2.3. Cell culture and differentiation

C2C12, mouse skeletal myoblast cell line, was maintained at sub-confluent densities (60–70%) in DMEM supplemented with 20% fetal calf serum (FCS), 100 units/ml of penicillin and 100 μg/ml of streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. To induce differentiation, cells at 80–90% confluence were shifted to DMEM supplemented with 2% horse serum, (differentiating medium (DM)). At different time-points of differentiation, cells were lysed in lysis buffer (50 mM Tris–HCl (pH 7.4), containing 50 mM NaCl, 5 mM EDTA, 1 mM PMSF, 50 mM NaF, 0.2 mM Na_3VO_4, 0.2% (v/v) NP-40 and protease inhibitor cocktail (PIC). For immuno-flourescence studies, cells were grown on cover-slips and allowed to differentiate for different lengths of time, as indicated. Cells were fixed with 3.7% (v/v) formaldehyde after each time-point and processed for confocal analysis. The differentiation index (DI) and fusion index (FI) was calculated as described by Sabourin et al. [22], using the following formulae;

$$\mathrm{DI} = \frac{\text{No. of myogenin positive cells}}{\text{No. of nuclei}} \qquad \mathrm{FI} = \frac{\text{No. of fused cells}}{\text{No. of nuclei}}$$

The differentiation and fusion indices were calculated as per the above formulae and represented in percentage.

2.4. Stable transfections

For stable transfections, C2C12 cells were grown in six well plate and transfected either with 1 μg pCDNA3 vector alone or with 1 μg of pCDNA3-N FLAG-tagged-αB-crystallin plasmids using Lipofectamine 2000 reagent (Invitrogen Corp., USA). At 48 h post-transfection, the cells were sub-cultured in medium containing geneticin (Roche Applied Sciences, USA) and grown for a period of one month. Subsequently, stably transfected clones of αB-crystallin were selected in the presence of 500 μg/ml of G418 (Geneticin) (Invitrogen Corp., USA). Single cell clones were isolated and the ones expressing not less than two fold higher levels of αB-crystallin (as determined by western blot analysis using anti-Flag antibodies) were expanded and used for our study. C2C12 cells that stably over-expressed αB-crystallin were referred to as CRYAB-C2C12.

2.5. FACS analysis

C2C12 cells and CRYAB-C2C12 cells were allowed to differentiate in DMEM medium containing 2% horse serum and harvested at different time-points of differentiation. At each time point, the cells were fixed in 80% methanol, stained with propidium iodide, and cell cycle analysis was done using Fluorescence Activated Cell Sorter, (Facs Caliber-Becton and Dickinson USA). The values represent average of three independent experiments and are expressed as percentage.

2.6. Immunofluorescence microscopy

C2C12 cells and cells over-expressing αB-crystallin (CRYAB-C2C12) were grown on cover slips till 80–90% confluence. Subsequently, cells were shifted to differentiating medium (DM) and allowed to undergo differentiation for different time periods. At each time point, cells were washed twice with ice-cold PBS and fixed with 3.7% formaldehyde. The fixed cells were permeabilized with 0.05% Triton X-100 for 8 min. After blocking with 2% BSA, cells were incubated with antibodies specific for myogenin and αB-crystallin, followed by incubation with Alexa-488- and Cy3-tagged secondary antibodies respectively. The cells were mounted in Vectashield medium containing DAPI. Confocal laser scanning microscopy was performed on a Carl Zeiss inverted microscopy. Image analysis was done using LSM 510 Meta software (Version 5).

2.7. SDS-PAGE and Western blot analysis

C2C12 cells and CRYAB-C2C12 cells were induced to undergo differentiation in DM and harvested at respective time points. Cells were lysed in ice-cold lysis buffer, sonicated and centrifuged at 20, 800 g for 10 min at 4 °C. Equal amount of protein was loaded on 12% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membrane using a semi-dry transfer apparatus (Amersham Pharmacia). The membrane was blocked with 10% (w/v) milk protein and incubated sequentially with appropriate primary antibodies and HRPO-conjugated secondary antibodies and was visualized using ECL kit (Amersham Biosciences, USA) according to the manufacturer's instructions. The band intensities were quantified by densitometry using GeneTools software (Syngene). The ratio of band intensities of the respective blots and that of the corresponding loading control, expressed in arbitrary units was used for comparison. The values represent an average of a minimum of three independent experiments.

2.8. Comparison of protein synthesis rate

C2C12 cells and CRYAB-C2C12 cells were allowed to differentiate for 12 h and 24 h respectively and subsequently incubated with MG132 (10 μM) to inhibit the 26S proteasomal degradation pathway.