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S100B in myoblasts regulates the transition from activation to quiescence and from quiescence to activation and reduces apoptosis ☆

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ABSTRACT

S100B protein activates IKKβ/NF-κB within myoblasts, thereby inhibiting the expression of MyoD and the MyoD-downstream effectors, myogenin and $p21^{WAF1}$, and myoblast differentiation. Herein we show that myoblasts downregulate S100B expression once transferred from proliferation medium to differentiation medium via a p38 MAPK-driven transcriptional mechanism as well as a post-translational, proteasome-dependent mechanism, and that myoblasts that have not been committed to differentiation resume expressing S100B once transferred back to proliferation medium. Likewise, myoblasts downregulate S100B expression once transferred to quiescence medium, and interference with S100B downregulation as obtained by stable overexpression of the protein results in reduced acquisition of quiescence and a faster proliferation upon transfer of the cells from quiescence medium to proliferation medium, compared to controls. These latter effects are dependent on S100B-induced activation of JNK. Moreover, S100B reduces myoblast apoptosis in an MEK-ERK1/2, Akt, JNK, and NF-κB-dependent manner. However, myogenin^{+} myoblasts (i.e., myocytes) and myotubes abundantly express S100B likely induced by myogenin. Our results suggest that (1) a timely repression of S100B expression is required for efficient myogenic differentiation; (2) S100B plays an important role in the expansion of the activated (i.e., proliferating) myoblast population; (3) under conditions associated with enhanced expression of S100B, the transition from proliferation to quiescence and from quiescence to proliferation might be altered; and (4) S100B exerts different regulatory effects in myoblasts and myocytes/myotubes/myofibers. This article is part of a Special Issue entitled: 11th European Symposium on Calcium.

1. Introduction

S100B is a Ca^{2+}-modulated protein of the EF-hand type that acts both as an intracellular regulator and an extracellular signal [1]. Intracellular S100B has been involved in the regulation of cell cycle progression and modulation of cell differentiation. Indeed, elevated levels of S100B are detected in several malignant tumors [2,3]; S100B negatively regulates the expression and function of the tumor suppressor p53 [4]; forced expression of S100B in the PC12 neuronal cell line results in enhanced proliferation and reduced responsiveness to the differentiating effect of nerve growth factor [5]; expression of S100B modulates chondrocyte terminal differentiation [6]; and repression of S100B expression in astrocyte cell lines and primary astrocytes results in reduced proliferation and migration and enhanced differentiation [7].

Myogenesis is a multistep process by which mesenchymal cells that are committed to myoblasts migrate to body sites of skeletal muscle formation, proliferate, and eventually fuse with each other to form myotubes, the ultimate precursors of skeletal muscle fibers [8–10]. Similarly, in the adult skeletal muscle tissue that has undergone damage mononucleated, quiescent satellite cells that coexist with myofibers and represent the adult stem cell pool of muscle tissue become activated, emigrate, proliferate, and eventually fuse with each other to form new myofibers and/or fuse with the damaged myofibers to repair them [8–10]. The regulation of myoblast and satellite cell proliferation and differentiation relies on the activity of extracellular factors such as insulin and insulin-like growth factors, basic fibroblast growth factor, hepatocyte growth factor, leukemia inhibitory factor, myostatin, transforming growth factor-β, members of the Wnt family, and several cytokines. These differentially govern myoblast and satellite cell migration, proliferation, and/or differentiation by affecting the activity of several intracellular pathways as well as the activity of both muscle-specific (i.e., Myf5, MyoD, myogenin, and MRF4) and non-specific transcription factors [8–10]. Among these, myogenin plays a crucial role, as its expression is indispensable for myogenic differentiation and marks the transition from myoblasts to myocytes, i.e., cells that have irreversibly exited the cell cycle and are fusion-competent [8–10]. Among the muscle non-specific transcription factors NF-κB appears to play a fundamental role both in physiological and pathological conditions [11–20].

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We have reported that administration of S100B to myoblasts in differentiation medium (DM) results in inhibition of differentiation, stimulation of proliferation, and protection against apoptosis via inactivation of the promyogenic p38 MAPK and activation of the mitogenic ERK1/2 [21,22]. However, S100B is expressed in myoblast cell lines [23] and in muscle satellite cells in situ [24], raising the possibility that intracellular S100B might have a role in myoblast proliferation/survival and/or differentiation. Indeed, increasing intracellular S100B levels resulted in a dramatically reduced myogenic differentiation via IKKβ/NF-κB-dependent inhibition of the expression of the myogenic transcription factor, MyoD, and its downstream effectors, myogenin and $p21^{WAF1}$, while inhibition of S100B expression resulted in a significant acceleration of differentiation as a result of reduction of NF-κB activity and consequent upregulation of MyoD [24].

During muscle regeneration/repair, a fraction of activated satellite cells neither participate in the formation of myotubes nor die by apoptosis; this fraction of cells stop proliferating and reacquire a quiescent status, thus reconstituting the pool of satellite cells [8–10]. The ability of S100B to modulate MyoD expression raised the possibility that the protein might have a role in the transition of myoblasts from proliferation to quiescence and/or the transition from quiescence to proliferation, given that downregulation of MyoD expression was shown to be required for proliferating myoblasts to become quiescent [25], and activation of quiescent myoblasts/satellite cells was shown to result in expression of MyoD [26–28]. We show here that (1) myoblasts in DM downregulate S100B mRNA and protein as a consequence of both reduction of serum mitogens and activation of the promyogenic p38 MAPK; (2) myoblast cultures resume expressing S100B mRNA expression between 24 and 48 h in DM; however, S100B protein levels remain low in non-fused $myogenin^{-}$ myoblasts due to proteasomal degradation while $myogenin^{+}$ myoblasts (i.e., myocytes) show highly abundant S100B; (3) increasing S100B levels in myoblasts results in a reduced acquisition of quiescence and an accelerated resumption of proliferation upon switching the cells from quiescence medium (QM) to growth medium (GM); and (4) enhancing S100B levels results in resistance to apoptotic stimuli. These findings suggest that downregulation of S100B is permissive for myogenic differentiation, and intracellular S100B might promote the expansion of myoblasts following their activation. The present results also suggest that conditions associated with elevated S100B levels in proliferating myoblasts might interfere with the homeostasis of the muscle satellite cell reserve pool.

2. Materials and methods

2.1. Cell culture

Wild-type (WT) L6 myoblasts, S100B-overexpressing L6 myoblasts (clone L6C8), and mock-transfected L6 myoblasts (clone L6C11) myoblasts were cultured for 24 h in DMEM supplemented with 10% fetal bovine serum (FBS) (growth medium—GM) (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin, in a H_2O-saturated 5% CO_2 atmosphere at 37 °C before decreasing FBS to 2% (differentiation medium—DM) to induce myoblast differentiation and myotube formation. For analyses of effects of S100B on the transition of myoblasts from proliferation to quiescence and from quiescence to proliferation, L6C11 and L6C8 myoblasts were cultivated in GM for 24–48 h, then transferred to methionine-depleted DMEM, 1% FBS (quiescence medium—QM) [26], and finally switched to GM. At each time point, the cultures were analyzed for proliferation rate by bromodeoxyuridine (BrdU) incorporation assay (see below). In some experiments, myoblasts were cultivated in the presence of the NF-κB inhibitor, pyrrolidine carbodithioic acid (PDTC) (Sigma) (10 μM), the PI3K inhibitor, LY294002 (Calbiochem) (10 μM), the p38 MAPK inhibitor, SB203580 (Calbiochem) (10 μM), the MEK-ERK1/2 inhibitor, PD98059 (Calbiochem) (30 μM), or the JNK inhibitor, SP600125 (Alexis, Lausen, Switzerland) (10 μM).

2.2. Transfections

S100B-overexpressing L6 myoblasts (clone L6C8) and mock-transfected L6 myoblasts (clone L6C11) were obtained as described [24], and S100B protein expression levels in L6C8 myoblasts were ~3 times higher than in L6C11 myoblasts [24]. L6C8 and L6C11 myoblasts were used in experiments as described above and below, in media containing G418 (200 μg/ml). WT L6 myoblasts were transiently transfected with myogenin expression vector or empty vector for 48 h in GM. The cells were cultivated for 24 h in DM and analyzed for S100B mRNA levels by real-time PCR or subjected to double immunofluorescence using a polyclonal anti-S100B antibody and a monoclonal anti-myogenin antibody (see below).

2.3. Western blotting

Myoblasts were solubilized with 2.5% SDS, 10 mM Tris–HCl, pH 7.4, 0.1 M dithiothreitol, 0.1 mM 0.1 mM tosylsulfonyl phenylalanyl chloromethyl ketone protease inhibitor (Roche). Cell lysates were subjected to Western blotting using the following antibodies: polyclonal anti-phosphorylated (Thr180/Tyr182) p38 MAPK (1:1000), a polyclonal anti-phosphorylated (Ser473) Akt (1:1000), polyclonal anti-p38 MAPK (1:2000) and a polyclonal anti-Akt (1:2000), polyclonal anti-phosphorylated (Thr202/Tyr204) ERK1/2 (1:2000) (all from Cell Signaling Technology), polyclonal anti-ERK1/2 (Sigma) (1:20,000), polyclonal anti-S100B (Epitomics) (1:1000), polyclonal anti-phosphorylated NF-κB (p65) (Cell Signaling Technology) (1:1000), polyclonal anti-NF-κB (p65) (Santa Cruz Biotechnology) (1:500), polyclonal anti-phosphorylated (Thr183/Tyr185) SAPK/JNK (1:1000; Cell Signaling Technology), and monoclonal anti-α-tubulin (Sigma) (1:10,000). The immune reaction was developed by enhanced chemiluminescence (SuperSignal West Pico, Pierce). Filters were subjected to densitometric analysis of the pertinent immune bands and their relative standard references (i.e., unphosphorylated kinases and NF-κB or tubulin) using SCION Image.

2.4. Immunofluorescence

S100B immunofluorescence analyses was performed as described [24] using a polyclonal anti-S100B antibody (SWant) (1:100). NF-κB (p65) and myogenin immunofluorescence analyses were performed as described for S100B using a polyclonal anti-NF-κB antibody (p65) (Santa Cruz Biotechnology) (1:1000) and a monoclonal anti-myogenin (Santa Cruz Biotechnology) (1:20). BrdU incorporation assay was performed by immunofluorescence. BrdU was added to cultures 2 h before fixation with cold methanol at −20 °C and processing by immunofluorescence using a monoclonal anti-BrdU antibody (1:50, Santa Cruz Biotechnology). $BrdU^{+}$ and total cells were counted. Nuclei were counterstained with DAPI. After mounting, the cells were viewed on a DM Rb epifluorescence microscope (Leica, Wetzlar, Germany) equipped with a digital camera. For comparative analyses, pictures were taken at a constant exposure time and gain in the same experimental setting, and ten random fields/coverslip were photographed each by two independent investigators.

2.5. Reverse transcriptase-PCR and real-time PCR

Total RNA was extracted using the TRIzol reagent (Gibco BRL) according to the manufacturer's instructions. The following primers were employed for reverse transcriptase (RT)-PCR: rat S100B, forward 5′GCCCTCATTGATGTCTTCCATC3′ and reverse 5′AAGAACTCATGACAGGCTGTGG3′; MyoD, forward 5′TGCAAGCGCAAGACCACTAA3′ and reverse 5′ACTGTAGTAGGCGGCGTCGTA3′; and rat GAPDH,