Bone 44 (2009) 53–60

Contents lists available at ScienceDirect

Bone

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

Arkadia represses the expression of myoblast differentiation markers through degradation of Ski and the Ski-bound Smad complex in C2C12 myoblasts

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ARTICLE INFO

Article history:
Received 28 July 2008
Revised 17 September 2008
Accepted 22 September 2008
Available online 7 October 2008

Edited by: T. Matsumoto

Keywords:
Myoblast differentiation
Arkadia
Degradation
Ski
Smad

ABSTRACT

The differentiation of myoblasts is regulated by multiple extracellular and intracellular factors. Of the extracellular regulators, members of transforming growth factor-β (TGF-β) family play critical roles in the regulation of osteoblasts and myoblast differentiation. Little is known, however, about the regulation of Myostatin/TGF-β signaling during myoblast differentiation. In this study, we examined the roles of Arkadia, an E3 ubiquitin ligase, in Myostatin/TGF-β signaling and the regulation of myoblast differentiation. Knockdown of Arkadia reduced Myostatin/TGF-β signaling and enhanced the differentiation of C2C12 myoblasts. In addition, exogenous overexpression of Arkadia enhanced Myostatin/TGF-β signaling, preventing myoblast differentiation. In the absence of the activation of Myostatin/TGF-β signaling, knockdown of Arkadia enhanced myoblast differentiation via upregulation of Ski protein, an intracellular enhancer of myoblast differentiation. Arkadia likely affected the differentiation of myoblasts in a Smad-independent fashion by inducing Ski degradation. Knockdown of Arkadia increased the Myostatin-induced phosphorylation of Smad2/3 in C2C12 cells. Arkadia bound Smad2/3 via Ski to induce the ubiquitination of Smad2/3. These results suggest that Arkadia targets Ski-bound, inactive phospho-Smad2/3 to regulate positively Myostatin/TGF-β signaling. Taken together, this study indicates that Arkadia regulates myoblast differentiation through both Smad-dependent and Smad-independent pathways.

Introduction

Myoblasts are derived from mesenchymal stem cells. The differentiation of myoblasts requires the upregulation of *MyoD* and *Myf5*, two basic helix–loop–helix transcriptional activators belonging to the myogenic regulatory factor (MRF) family [1]. These master regulators of myoblast differentiation are controlled by a variety of intracellular factors, including Ski, Smad, and extracellular signal-regulated kinase 1/2 (Erk1/2) [2–5]. Of these factors, Ski may play an important role in the positive regulation of skeletal muscle growth and differentiation. Ski was originally identified as the transforming oncogene of the avian Sloan–Kettering retrovirus [6]. *In vitro*, Ski induces myoblast differentiation [3,7], and Ski transgenic mice have greatly increased skeletal muscle mass [2].

The differentiation of myoblasts is also regulated by extracellular factors, such as members of the fibroblast growth factor (FGF) family, Myostatin [also termed growth/differentiation factor-8 (GDF-8)], transforming growth factor-β (TGF-β), insulin-like growth factor (IGF), and hepatocyte growth factor (HGF) [1]. Of these secreted factors, TGF-β and Myostatin play crucial roles in the negative regulation of skeletal muscle growth and differentiation [4,8–12]. Myostatin-deficient mice exhibit skeletal muscle hypertrophy and hyperplasty [10]; Myostatin mutations in cattle result in the double muscling phenotype [13,14]. Myostatin mutations have been noted to be associated with gross muscle hypertrophy in a child [15], while systemic overexpression of Myostatin leads to a human cachexia syndrome exhibiting prominent muscle loss [16].

Intracellular signaling by TGF-β family members is mediated by Smad proteins [17]. After stimulation of TGF-β or Myostatin, type I receptors phosphorylate the receptor-regulated Smads (R-Smads), Smad2 and Smad3. Phosphorylated Smad2 and Smad3 form heteromeric complexes with Smad4, the common-partner Smad (Co-Smad), and translocate into the nucleus to regulate the transcription of target genes [17,18]. Inhibitory Smads (I-Smads) are induced by multiple stimuli, including TGF-β, bone morphogenetic protein (BMP), and Myostatin, leading to the negative regulation of TGF-β family signaling [19,20]. Ski and SnoN, a Ski-related protein, are also involved in the negative regulation of Smad signaling through physical interactions

Abbreviations: TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; shRNA, short hairpin RNA; RT-PCR, reverse transcription-polymerase chain reaction.

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doi:10.1016/j.bone.2008.09.013

with Smad2, Smad3, and Smad4 [21]. As aberrations in TGF-β and Myostatin signaling have been reported to induce the progression of diseases, such as cancer and skeletal muscle disorders, TGF-β and Myostatin signaling must be tightly regulated. Despite the importance of these pathways, little is known about the regulation of Myostatin signaling.

The ubiquitin–proteasome system plays essential roles in a wide variety of fundamental biological processes, including cell-cycle progression, signal transduction, and transcriptional regulation [22,23]. Arkadia, a RING-type E3 ubiquitin ligase [24], was originally identified as an intracellular protein essential for the formation of a mammalian node during mouse development [25]. This protein is also an essential positive modulator of Nodal signaling, leading to the induction of Spemann's organizer [26]. We previously reported that Arkadia enhances TGF-β signaling via Smad7 degradation [24]. Recently, several groups, including us, have reported that Arkadia promotes the ubiquitination and degradation of Ski/SnoN as well as Smad7 [27,28]. As Arkadia-deficient mice die *in utero*, the physiologic role of Arkadia remains unclear.

Here we investigated the function of Arkadia and the molecular mechanisms by which the protein regulates Ski and Smads function during myoblast differentiation.

Materials and methods

Reagents

Recombinant human Myostatin was purchased from Peprotech (Rocky Hill, NJ). TGF-β3 was acquired from R&D systems (Minneapolis, MN). A-44-03, an inhibitor of ALK4/5/7 kinases, has been described previously [29]. MG132 was obtained from Peptide Institute (Osaka, Japan). The original constructs encoding Arkadia, Smad3, Ski, and ubiquitin (Ub) cDNAs have been described previously [24,27,30]. We used anti-tubulin (Sigma, St. Louis, MO), anti-Myosin heavy chain (MHC) (MF-20; Developmental Studies Hybridoma Bank, provided by Donald A Fishman, MD), anti-Smad7 (N19; Santa Cruz, Santa Cruz, CA), anti-Ski (G8; Santa Cruz), anti-phospho-Smad2 (Cell Signaling, Beverly, MA), anti-phospho-Smad3 (Cell Signaling), anti-Smad2/3 (Becton Dickinson, Franklin Lakes, NJ), anti-myc (9E10; Becton Dickinson), anti-HA (InvivoGen, San Diego, CA), and anti-Flag (M2; Sigma) antibodies where indicated. The anti-Arkadia 62 antiserum was previously described [24].

Cell culture and myoblast differentiation

C2C12, a murine myoblast cell line, was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Lowering the concentration of FBS to 2% induced the myoblastic differentiation of C2C12 cells. Human embryonic kidney 293 cells, 293T cells constitutively expressing the simian virus 40 (SV-40) T antigen, murine breast cancer 4T1 cells, and murine marrow stroma cells (MSCs) were maintained in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. ST2, a murine bone marrow stroma cell line, was maintained in RPMI-1640 medium (Sigma) containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Murine mammary epithelial NMuMG cells were maintained in DMEM containing 10% FBS, 10 μg/ml insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Quantitative real-time RT-PCR

For the *in vivo* experiments, total RNA was extracted from tissues of three day-old mice, including bone, skeletal muscle, brain, lung, heart, liver, and kidney. The extracted tissues were minced and homogenized in Trizol reagent (Invitrogen, Carlsbad, CA). For the *in vitro* experiments, total RNA was purified from cell lysates using Trizol reagent (Invitrogen). First-strand cDNA was synthesized using Prime Script reverse transcriptase (TaKaRa Bio, Shiga, Japan) with Oligo dT [12–18] as a primer. We performed quantitative reverse transcription-polymerase chain reaction (RT-PCR) using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). All experiments were repeated at least twice and all samples were run in duplicate in each experiment. Values were normalized to Hprt1 levels. The primers used are listed in Supplementary Table 1.

Generation of lentiviral vectors expressing shRNA

Lentiviruses carrying short hairpin (sh) RNA (LV-shRNA) sequences were generated from the CS-RfA-EG, pCAG-HIVgp, and pCMV-VSV-G-Rev plasmids. We subcloned the shRNA sequence for mouse Arkadia (5′-GAGTGGAGGTTAATATACTAC-3′) into the CS-RfA-EG Vector, which also bears a green fluorescent protein (GFP) expression cassette. A lentivirus encoding the control shRNA (5′-GCGCGCTTTGTAGGATTCG-3′) was also generated. After infection of C2C12 cell cultures with LV-shRNA at a multiplicity of infection (MOI) of 300, infected cells were isolated by fluorescence-activated cell sorting for positive GFP expression using a BD FACSArea cell sorter (Becton Dickinson).

Generation of adenoviruses

Adenoviruses were generated using an adenovirus expression vector kit (TaKaRa Bio) as described previously [31]. Recombinant adenoviruses carrying Flag-tagged Arkadia, Ski, or LacZ were constructed in 293 cells by homologous recombination of the expression cosmid cassette (pAxCAwt) and the parental virus genome. Viral titers were determined using an Adeno-X rapid titer kit (TaKaRa Bio). Infection of C2C12 cells with recombinant adenoviruses was performed by incubation at an MOI of 300.

Immunoprecipitation and immunoblotting

293T cells were transiently transfected with plasmids using FuGENE6 (Roche Diagnostics, Indianapolis, IN) for 24 h before analysis. To inhibit proteasomal degradation, cells were incubated for 4 h with 10 μM MG132. To stimulate the TGF-β signaling, cells were incubated for 4 h with 0.3 ng/ml TGF-β3. 293T cells were lysed in buffer containing 1% Igepal CA-630, 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). C2C12 cells were harvested in RIPA buffer containing 1% Triton-X100, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% sodium lauryl sulfate (SDS), 0.5% sodium deoxycholate, 1% aprotinin, and 1 mM PMSF. Lysates were sonicated and centrifuged. We determined the concentrations of protein in each supernatant using a DC Protein Assay kit (Bio-Rad Laboratories, Boston, MA). Immunoprecipitation and immunoblotting were performed as described previously [32]. When indicated, immunoblotting was performed using an LAS-3000 mini lumino-image analyzer (Fuji film, Tokyo, Japan) instead of X-ray film.

Statistical analysis

The results of the quantitative real-time RT-PCR experiments were expressed as means ± standard errors of the means. Statistical significance was determined using the two-sided Student's *t*-test.

Results

Knockdown of Arkadia reduces Myostatin and TGF-β signaling and enhances myoblastic differentiation

Arkadia positively regulates TGF-β family signaling [24,27,33]. The molecular mechanisms by which Arkadia functions and the resulting