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Enhancement of C2C12 myoblast proliferation and differentiation by GASP-2, a myostatin inhibitor

Luce Pèrié, Alexis Parenté, Caroline Brun [1], Laetitia Magnol, Patrick Pélissier, Véronique Blanquet *

Univ. Limoges, INRA, UMR 1061, UGMA, F-87000 Limoges, France

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ABSTRACT

Background: GASP-2 is a secreted multi-domain glycoprotein known as a specific inhibitor of myostatin and GDF-11. Here we investigate the role of GASP-2 on myogenesis and the effect of its glycosylation on its activity.
Methods: GASP-2 overexpression or knockdown by shRNAs were carried out on C2C12 myoblasts cells. *In silico* analysis of GASP-2 protein was performed to identify its glycosylation sites. We produced a mouse recombinant GASP-2 protein in a prokaryotic system to obtain a fully deglycosylated protein allowing us to study the importance of this post-translational modification on GASP-2 activity.
Results: Both mature and deglycosylated GASP-2 proteins increase C2C12 proliferation and differentiation by inhibiting the myostatin pathway. *In silico* and western-blot analyses revealed that GASP-2 presents one consensus sequence for *N*-glycosylation and six potential sites of mucin-type *O*-glycosylation.
Conclusions: GASP-2 promotes myogenesis and thus independently of its glycosylation.
General significance: This is the first report demonstrating that GASP-2 promotes proliferation and differentiation of myoblasts by inhibiting the canonical pathway of myostatin.

1. Introduction

GASPs (Growth and Differentiation Factor Associated Serum Protein) are secreted glycoproteins known to interact with myostatin (MSTN), a key regulator of muscle development [1,2]. These proteins contain multiple protease inhibitors domains [3,4]. Thus, the two members, GASP-1 and GASP-2 (also named WFIKKN2 and WFIKKN1) are made of four serine proteases inhibitor modules (WAP, Follistatin/Kazal and two Kunitz), a Netrin domain which is implicated in inhibition of metalloproteinases and an Ig domain involved in protein folding (Igc2) [3,4]. These domains are highly conserved among mammals especially the Follistatin/Kazal, the second Kunitz and the Netrin domains [5]. This conservation strongly suggests the importance of their functions [5]. Since 2003 and its discovery [6], most of the studies have focused on the role of GASP-1 as a myostatin antagonist. It was shown that GASP-1 interacts with and inhibits myostatin *via* its Follistatin domain [6–9]. The myostatin is a member of TGFβ superfamily that negatively regulates myogenesis mainly *via* the SMAD2/3 pathway [10]. The disruption of the *MSTN* gene in mice leads to a dramatic increase of skeletal muscle mass due to both hyperplasia and hypertrophy [1]. Overexpression of myostatin inhibitors, like Follistatin and FSTL3, leads to an increase of myoblasts proliferation and differentiation and an increase of muscle mass in mice [11,12]. Recently, we also described in mice overexpressing *Gasp-1* a hypermuscular phenotype owing only to hypertrophy, without hyperplasia of the myofibers [13]. We demonstrated that in addition to the inhibition of its canonical pathway, myostatin is up-regulated in these mice leading to the absence of hyperplasia [14].

Unlike GASP-1, GASP-2 was never found associated with myostatin in serum although their interaction is well known [8]. As GASP-1 and GASP-2 share 54% of identity and are expressed, at few exceptions, in similar tissues including skeletal muscle [4], we asked if GASP-2 could also be involved in the regulation of myostatin during myogenesis. In this paper, we analyzed at cellular and molecular levels the consequences on myoblasts proliferation and differentiation of *Gasp-2* over or knockdown gene

* Correspondence to: Université de Limoges, UMR 1061, Unité de Génétique Moléculaire Animale, Faculté des Sciences et Techniques, 123, av. A. Thomas, F-87060 Limoges Cedex, France.
E-mail addresses: luce.perie@etu.unilim.fr (L. Pèrié), alexis.parente@etu.unilim.fr (A. Parenté), cbrun@ohri.ca (C. Brun), laetitia.magnol@unilim.fr (L. Magnol), patrick.pelissier@unilim.fr (P. Pélissier), veronique.blanquet@unilim.fr (V. Blanquet).
[1] Present address: Sprott Centre for Stem Cell Research, Ottawa Health Research Institute, ON K1H 8L6 Ottawa, Canada.

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expression. Like most of secreted proteins, GASP-2 is glycosylated. In addition to play a central role on the solubility and half-life of the protein, glycosylation is well known to modulate its function. We also analyzed the involvement of this post-translational modification on GASP-2 activity, focusing our study on its role as an inhibitor of myostatin.

2. Materials and methods

2.1. Cell culture

Mouse C2C12 myoblasts [15] were obtained from American Type Culture Collection (ATCC). C2C12 myoblasts were grown in Growth Medium (GM) consisting in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen), 100 units ml^{-1} penicillin and 100 µg ml^{-1} streptomycin (Invitrogen). C2C12 differentiation was induced upon 70% confluence by serum withdrawal (DMEM with 2% horse serum (HS, Invitrogen)). For proliferation assay, C2C12 were grown or not with 1 µg ml^{-1} of hGASP-2 or mGASP-2 deglycosylated (deglyco-mGASP-2).

2.2. Bioinformatics analyses

GASP-2 orthologs were retrieved from databases using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and Ensembl Genome Browser (www.ensembl.org). Alignment was performed using ClustalW program and analyzed with WebLogo. Asparagine and serine/threonine residues potentially glycosylated were identified using respectively NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 3.1 Server [16].

2.3. Transient transfections of C2C12 cells

Mouse C2C12 myoblasts at 70% confluence were transiently transfected using the XtremeGENE 9 Transfection Reagent (Roche Applied Science) with 5 µg of pcDNA3.1-Gasp-2 or pcDNA3.1-empty in DMEM serum-free media. After 16 h of transfection, the medium was replaced and myoblasts were allowed to proliferate and differentiate for further analyses.

2.4. Production and purification of murine GASP-2 in prokaryotic system

The mouse *Gasp-2*cDNA coding sequence without peptide signal were amplified and cloned into the pGEX-4-T1 plasmid (Sigma Aldrich). Production was carried out in *E.coli* BL21 (DE3) in LB (Luria Bertani) supplemented with 0.2% glucose and 100 µg ml^{-1} ampicillin. Bacteria were grown to reach OD_{600} at 0.5 and then induced with 0.025 mM IPTG at 20 °C for 14 h at 250 rpm. Bacterial growth was stopped by centrifugation (6000 g, 15 min; 4 °C). Bacteria were lysed in 20 ml of lysis buffer (20 mM Tris–HCl, pH7.5, 250 mM NaCl, 1 mM EDTA, 0.05% Triton X-100, 10 µg ml^{-1} lysozyme) and centrifuged (48,000*g*, 20 min, 4 °C). Purification was carried out with the ÄKTAprime system (Amersham-Biosciences). Supernatant was loaded into a GST-Trap HP 5 ml column (GE Healthcare) equilibrated in 20 mM sodium phosphate, pH7.3, 0.15 M NaCl. Elution was performed at a rate of 1 ml min^{-1} with 50 mM Tris–HCl, pH8, 10 mM reduced glutathione. Cleavage of the GST Tag located at the N-terminal region of the fusion protein was performed with 10 units mg^{-1} of recombinant thrombin protein (Sigma T68884) by incubation for 16 h at 4 °C. The sample was then concentrated on an Amicon Ultra 30 K filter (Millipore) by centrifugation (4000*g*, 4 °C).

2.5. Quantitative real-time PCR (qPCR)

Total RNA from cells and tissues was isolated using RNeasy midi kit (Qiagen). Synthesis of cDNA was performed with the High Capacity cDNA Archive Kit (Applied Biosystems) to convert 2 µg of total RNA into single-stranded cDNA. Real-time PCR was performed in triplicate using 50 ng of cDNA. Relative amounts of transcripts were determined using Taqman probes specific for *Gasp-2* (Mm01308311_m1), *β2m* (Mm00437762_m1), *Dffa* (Mm00507317) and *MyoG* (Mm00446194_m1), on an ABI PRISM© 7900 System. Relative mRNA expression values were calculated by the ΔΔCt method with normalization of each sample to the average change in cycle threshold (Ct) value of the controls. For all analyses, three independent experiments have been performed, each assay corresponding to 3 wells /condition/ probe.

2.6. Proliferation assay

Three independent experiments have been carried out. Each experiment corresponds to the analysis of 12 wells with 2000 cells/well at t=0 h. The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) was used to evaluate the cell proliferation during 72 h. 20 µl de MTS solution was added to the cells for 3 h and absorbance at 490 nm was then recorded with a 96-well plate reader.

2.7. Fusion index

The fusion index corresponds to the proportion of nuclei present within myotubes that contain two or more nuclei. Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) in PBS for 15 min. The fusion index was analyzed after hematoxylin and eosin (H&E) staining.

2.8. Lentiviral-mediated knockdown of Gasp-2

Individual shRNA constructs specifically designed to target the *Gasp-2* were purchased from Sigma Aldrich (Gasp-2_sh1: XM_128578.3-784s1c1; Gasp-2_sh2: XM_128578.3-500s1c1). Lentiviral particles, consisting of the shRNA transfer vector PLKO.1-puro (which contains the sequence of shRNA as well as the cis acting sequences necessary for RNA production and packaging) and the mission lentiviral packaging (Sigma Aldrich). Mission lentiviral particles were generated from different components. The packaging vector contains the minimal set of lentiviral genes required to generate the virion structural proteins and packaging functions. The pCMV-VSV-G envelope vector provides the envelope vector for pseudo-typing. The PLKO.1-puro *Gasp-2* shRNA or empty pLKO.1 and pCMV-VSVG vectors were transfected into HEK293Tcells using a Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. Briefly, HEK293T cells were grown in GM at 70-80% confluence and were transfected with 1 µg of plasmid PLKO.1-puro with the mission lentiviral packaging mix. After 16 h of transfection, the medium was replaced with fresh GM and cells were incubated for a further 60 h. The supernatant was then collected as a source of viral particles. C2C12 myoblasts were infected with lentivirus-containing media for 24 h and selected with 2 µg ml^{-1} puromycin.

2.9. SDS-page and western blot analyses

Cells and tissues were collected in lysis buffer (50 mM Tris, pH8, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, and protease inhibitors) followed by centrifugation at 12,000 g, 20 min, 4 °C. Protein quantification was performed with a Bradford assay. Proteins were separated under denaturing conditions into a