Experimental Cell Research 341 (2016) 187–195

Contents lists available at ScienceDirect

Experimental Cell Research

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

Research Article

Inhibition of the myostatin/Smad signaling pathway by short decorin-derived peptides

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ARTICLE INFO

Article history:
Received 13 November 2015
Received in revised form
27 January 2016
Accepted 31 January 2016
Available online 1 February 2016

Keywords:
Decorin
Small leucine rich proteoglycans
Myostatin
Peptide
TGF-β superfamily

ABSTRACT

Myostatin, also known as growth differentiation factor 8, is a member of the transforming growth factor-beta superfamily that has been shown to play a key role in the regulation of the skeletal muscle mass. Indeed, while myostatin deletion or loss of function induces muscle hypertrophy, its overexpression or systemic administration causes muscle atrophy. Since myostatin blockade is effective in increasing skeletal muscle mass, myostatin inhibitors have been actively sought after. Decorin, a member of the small leucine-rich proteoglycan family is a metalloprotein that was previously shown to bind and inactivate myostatin in a zinc-dependent manner. Furthermore, the myostatin-binding site has been shown to be located in the decorin N-terminal domain.

In the present study, we investigated the anti-myostatin activity of short and soluble fragments of decorin. Our results indicate that the murine decorin peptides DCN48-71 and 42-65 are sufficient for inactivating myostatin in vitro. Moreover, we show that the interaction of mDCN48-71 to myostatin is strictly zinc-dependent. Binding of myostatin to activin type II receptor results in the phosphorylation of Smad2/3. Addition of the decorin peptide 48-71 decreased in a dose-dependent manner the myostatin-induced phosphorylation of Smad2 demonstrating thereby that the peptide inhibits the activation of the Smad signaling pathway. Finally, we found that mDCN48-71 displays a specificity towards myostatin, since it does not inhibit other members of the transforming growth factor-beta family.

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1. Introduction

Despite adequate nutritional intake, the loss of skeletal muscle mass is observed in different pathological and non-pathological situations: in sarcopenia resulting from aging, or cachexia which is a wasting syndrome observed in patients with cancer, AIDS, chronic obstructive lung disease, muscular dystrophies such as Duchenne muscular dystrophy (DMD), etc. The important impact of skeletal muscle wasting is exemplified by the fact that it contributes to 20% of cancer deaths [1].

The mechanisms regulating skeletal muscle mass have been identified by studying among others the cattle breeds Belgian Blue and Piedmontese, which display an exceptional muscle development commonly referred to as "double-muscling" phenotype [2,3]. Mutations in the gene of *myostatin* (MSTN) are responsible for the increase of the muscle mass in these cattles and have also been found in hyper-muscled sheeps [4] and dogs [5,6]. In addition, a loss-of-function mutation in the myostatin gene has been identified in a child that exhibited a large increase in muscle mass and strength [7]. Altogether, the results show that myostatin is a key regulator of skeletal muscle mass. While its deletion or loss of function induces muscle hypertrophy, its overexpression or systemic administration causes muscle atrophy [8,9].

Myostatin is also referred to as growth differentiation factor-8 (GDF-8), and belongs to the transforming growth factor-beta (TGF-β) superfamily. The MSTN gene is highly conserved across species, since the sequences of murine, rat, human, porcine, chicken, and

Abbreviations: ActRIIB, activin receptor IIB; DCN, decorin; ECM, extracellular matrix; ED50, median effective dose; FAM, carboxyfluorescein; GDF-8, growth differentiation factor 8; GDF-11, growth differentiation factor 11; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MSTN, myostatin; SLRP, small leucine-rich proteoglycan; TGF-β, transforming growth factor-beta

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http://dx.doi.org/10.1016/j.yexcr.2016.01.019
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turkey myostatin are 100% identical in the C-terminal region [3]. Myostatin, which is predominantly expressed in skeletal muscle, is synthesized as pre-pro-myostatin. It is secreted as an inactive pro-myostatin, whose proteolytic cleavage generates an amino-terminal propeptide (28 kDa) and a carboxy-terminal MSTN (12.5 kDa) that is biologically active as dimer. However, even after cleavage, most of the mature MSTN remains bound to the propeptide – and forms the so-called latency associated complex – preventing MSTN binding to its receptor [9,10]. Activation of the latent complex releases the mature myostatin and allows its binding to activin type II receptors (primarily ActRIIB), which initiates an intracellular signaling cascade leading to the phosphorylation and activation of Smad2/3 [11]. The activated Smad complexes then translocate into to the nucleus where they modify the transcription of genes involved in cellular differentiation and proliferation. Myostatin also decreases Akt phosphorylation and signals through FOXO transcription factors, resulting in increased expression of atrophy-related genes and atrophy induction [12].

Considering MSTN negative effect on muscle growth, its inhibition has been proposed as a therapeutic approach in muscle-degenerative and wasting conditions, such as muscular dystrophies and cachexia. Several different anti-myostatin approaches have been developed in the last years, in particular inhibitors like MSTN propeptide [13,14], a soluble form of the activin receptor [15,16] and follistatin [17,18]. A clinical trial involving patients with muscular dystrophies has been performed using an anti-myostatin antibody (MYO-029 antibody) [19], but the results were not as good as expected and the trial was stopped. Thus, research on the development of new inhibitors of myostatin is still needed.

Decorin (DCN) is the best characterized member of the small leucine-rich proteoglycan (SLRP) family [20–22]. It has a core protein of 40 kDa and a single glycosaminoglycan chain with an averaged molecular weight of 22,000 Da covalently attached to a serine residue in the N-terminal part of the protein (Ser^{34}) (Fig. 1). After synthesis, DCN is secreted into the extracellular space where it participates to the organization of the extracellular matrix (ECM) through the interaction with a variety of matrix components including fibronectin and types I, IV, and V collagen [23]. In addition to the interaction with ECM constituents, decorin interacts with fibrinogen [23] as well as with growth factors such as TGF-β [24] and the connective tissue growth factor (CTGF/CCN2) [25]. Finally, decorin interacts also with different types of membrane-located receptors including the epidermal growth factor receptor [26], the insulin growth factor-1 receptor [27], Met which is the receptor for the hepatocyte growth factor [28] and the low density lipoprotein receptor-related protein (LRP-1) [29–31]. Altogether, the capacity of decorin to interact with such a variety of factors and receptors explains how this SLRP can be involved in mechanisms as different as wound healing [32], hepatic fibrosis [33], post-myocardial infarction remodeling [34], and suppression of tumorigenic growth and angiogenesis [35].

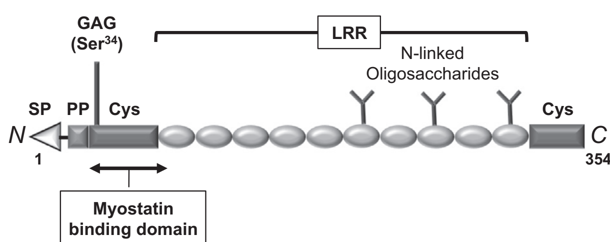

Fig. 1. Scheme of the decorin protein. A signal peptide (SP) composed of the first 16 residues is followed by a 14 amino acid long pro-peptide (PP). The protein core consists of leucine-rich repeats (LRR) with conserved Cys-rich N- and C-terminal domains. The N-terminal cysteine cluster has a CX3CXCX6C pattern, which is conserved in class I of the SLRPs. A single glycosaminoglycan (GAG) side chain is attached to serine 34. The binding domain to myostatin is located in the N-terminal domain.

Decorin, as other SLRPs such as biglycan, play also an important role in myogenesis [36,37]. Indeed, it was shown that decorin is able to activate the differentiation of skeletal muscle cells [38]. These results can be explained, at least in part, by the suppression of myostatin activity after binding of decorin to mature myostatin in the presence of zinc [39]. Also, it was shown that the direct injection of recombinant DCN efficiently prevents fibrosis and enhances muscle regeneration [40,41]. Based on these findings, we have recently shown that the intramuscular injection of recombinant decorin results in a significant increase of dystrophic muscle mass [42]. In addition, we have identified fragments of the DCN N-terminal region, in particular murine DCN peptide 31-71, that bind to and inactivate myostatin [42]. However, this latter peptide is prone to aggregation and is thus not easy to handle. Also, knowing that decorin can bind to different proteins including to TGF-β [24,43] the issue of the specificity of action of decorin fragments remained unanswered.

The three main objectives of the present study were to: i – identify short and well-soluble decorin-derived peptides that are able to inhibit myostatin, ii – characterize the mechanism of inhibition, and iii – demonstrate that the peptides have a specificity towards myostatin and do not inhibit other members of the TGF family, in particular TGF-β, GDF-11 and activin A.

2. Materials and methods

2.1. Materials

The recombinant mouse myostatin was obtained from R&D Systems. The branched polyethylenimine of 25 kDa (B-PEI) was obtained from Sigma-Aldrich. The DMAPAP cationic lipid was obtained from Dr. V. Escriou (UMR8258, Paris Descartes, France). The $p(CAGA)_{12}$-Luciferase reporter expression cassette was kindly given by Dr. C.H. Heldin.

2.2. Peptides

Peptides were synthesized by Proteogenix and GeneCust. The sequences of the different peptides are shown in Table 1. The 5-carboxyfluorescein (5FAM)-labeled peptides were modified at the N-terminus.

2.3. Cell culture

The human embryonic kidney cell line HEK293T cells (ATCC, CRL-1573) was cultured at 37 °C with 5% CO_2 using Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum (PAA).

Table 1
List of the mDecorin derived peptides.

Peptide	Sequence[a]
mDCN42-65	DNPLISMCPYRCQCHLRVVQCSDL
mDCN48-71	MCPYRCQCHLRVVQCSDLGLDKVP
Mutated mDCN48-71	M**A**PYR**A**Q**A**HLRVVQ**A**SDLGLDKVP[b]

[a] The peptides were amidated at the C-terminus and the cluster of cysteines are highlighted in gray;
[b] The alanine residues (in bold and underlined) were used to replace the amino acid cysteine of the wild-type sequence of the mDCN48-71 peptide.

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