Regulation of myostatin activity by WFKKN proteins

Ph. D. thesis

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Introduction

Human skeletal muscle is responsible not only for changing place or position of the entire body. It also plays a critical role in energy homeostasis. By outstanding plasticity it responds to changes in the amount of available calorie and physical activity. Skeletal muscle cells produce a range of myokine factors, which act on the functions of distant organs through the blood circulation. One of the most intensively researched myokine is the GDF8 (Growth and Differentiation Factor), better known as myostatin, a negative regulator of muscle mass.

Myostatin controls numerous biological processes ranging from muscle growth and development, through muscle regeneration, to brown fat development and energy homeostasis. Myostatin signalling is a promising therapeutic target in the fight against muscle wasting syndromes, obesity and type 2 diabetes. Myostatin regulates human characteristics that have a high impact on quality of life and longevity.

Two of the numerous myostatin antagonists, the WFIKKN1 (WAP Follistatin Immunoglobulin Kunitz Kunitz NTR containing protein) and WFIKKN2 were identified in the human genome by the Functional Genomics workgroup of the Institute of Enzymology in Hungary. The myostatin inhibitory function of WFIKKN2 was evinced by Jennifer Hill and associates during an assay for finding new binding partners of myostatin in mouse blood serum. I began my research by investigating the interactions between the WFIKKN2 protein and members of the TGFβ growth factor family including myostatin. The successful
production of recombinant human promyostatin in *E. coli* expression system allowed to study the interactions of different maturation forms of myostatin with the WFIKKN proteins. I also examined the effect of K153R mutation on myostatin function.

**Objectives**

- Determining the strength of the interactions between WFIKKN2 and members of the TGFβ family.
- Identifying differences between the biological functions of the WFIKKN proteins.
- Production of human promyostatin
- Characterization of the interactions between WFIKKN proteins and unprocessed myostatin forms
- Biochemical characterization of K153R mutant promyostatin

**Applied methods**

Recombinant WFIKKN proteins used in the experiments were produced in *Drosophila* S2 cells. Secreted WFIKKN proteins were harvested from the culture medium and were purified using Ni-NTA affinity and size exclusion chromatography. Active forms of TGFβ growth factors were purchased from commercial source. Wild type and K153R mutant form of promyostatin and myostatin prodomain were produced in *E. coli* BL-21 (DE3) expression system. The proteins were
refolded from inclusion bodies and purified by Strep-Tactin affinity and size exclusion chromatography.

The latent myostatin complex was produced by digestion of promyostatin with the proprotein convertase furin. Activation of the latent myostatin complex was achieved by BMP1 metalloproteinase digestion or by 80 °C heat treatment. Studies on the activity of myostatin preparations were carried out in a cell based reporter system. In the assay set up a luciferase encoding reporter construct equipped with a myostatin sensitive promoter was transiently transfected into A204 rhabdomyosarcoma cells. Luciferase activity was detected with an Enspire luminometer.

For determining the effectiveness of furin and BMP1 digestion of both the wild type and K153R mutant promyostatin and latent myostatin substrate, digested samples were run on 13.5% SDS polyacrylamide gels. The separated samples were visualized with Sypro Ruby fluorescent protein stain.

Utilizing the His6 affinity-tag on the recombinant WFIKKN proteins, I was able to study the interactions between WFIKKN proteins and partially processed forms of myostatin in pull-down assays. The interacting partners of the WFIKKN proteins were bound to a Ni-NTA resin, and the eluted samples were analysed on 13.5% SDS polyacrylamide gels. Visualisation of the separated samples were done either by protein staining or by western blot technique using myostatin growth factor domain and prodomain specific antibodies.
Interactions of WFIKKN2 protein with members of the TGFβ growth factor family were detected by surface plasmon resonance measurements on a Biacore X-100 instrument. Growth factor receptor binding, interactions of WFIKKN proteins with promyostatin and the interactions of WFIKKN proteins with myostatin prodomain were also studied using this technique. During sensor surface preparation one of the interacting partners was immobilized to the CM5 sensor surface using the amine coupling method. Kinetic parameters of the interactions were determined with the Biaevaluation 4.0 program.

Results

1. WFIKKN2 is a specific and potent inhibitor of myostatin and GDF11.

1.1. According to results of SPR measurements WFIKKN2 binds strongly to myostatin and GDF11 growth factors. The determined dissociation constant of the WFIKKN2-myostatin complex is $2.8 \times 10^{-10}$ M, whereas the dissociation constant of the WFIKKN2-GDF11 complex is $1.6 \times 10^{-10}$ M.

1.2. WFIKKN2 protein interacts with other members of the TGFβ growth factor family, but these interactions are typically two to three orders of magnitude weaker than the interactions between WFIKKN2 and myostatin or GDF11 ($\text{BMP2} - K_d = 4.3 \times 10^{-8}$ M, $\text{BMP3} - K_d = 1.8 \times 10^{-7}$ M, $\text{BMP4} - K_d = 6.5 \times 10^{-8}$ M, $\text{BMP8b} - K_d = 5.3 \times 10^{-5}$ M, $\text{TGFβ1} - K_{d1} = 2.8 \times 10^{-8}$ M, $K_{d2} = 3.3 \times 10^{-5}$ M).
1.3. SPR solution competition assays showed that WFIKKN2 effectively inhibits the receptor binding of the myostatin and GDF11 growth factors. The determined IC\textsubscript{50} values are 12 nM for myostatin inhibition and 5 nM for GDF11 inhibition. In the case of BMP2 and BMP4 growth factors only much higher concentrations of WFIKKN2 had a substantial negative effect on receptor binding. The IC\textsubscript{50} value for the BMP2 inhibition by WFIKKN2 is 2 μM (by 15 nM BMP2 concentration), whereas the IC\textsubscript{50} value for the BMP4 inhibition by WFIKKN2 is 3 μM (by 25 nM BMP4 concentration).

2. Production of recombinant human promyostatin in *E. coli* expression system.

2.1. The promyostatin dimer was refolded from inclusion body. Digestion of promyostatin with the proprotein convertase furin led to the formation of latent myostatin. The biologically active myostatin could be liberated from latent myostatin by BMP1 metalloproteinase digestion or 80 °C heat treatment.

3. Semilatent myostatin is capable of receptor binding

3.1. The reporter assay results and SPR measurements showed that latent myostatin preparations have significant activity. The receptor binding activity is due to the dissociated forms present in the latent myostatin preparation. The semilatent myostatin, in which only one prodomain complexes the growth factor dimer, is an existing dissociation intermediate capable of receptor binding.
4. Interactions between WFIKKN proteins and intermediate forms of myostatin processing.

4.1. Contrary to WFIKKN1, WFIKKN2 does not bind the prodomain of myostatin according the results of the SPR measurements. The WFIKKN1 binding site on the myostatin prodomain is located outside the growth factor binding N-terminal region.

4.2. WFIKKN1 binds promyostatin two orders of magnitude weaker than the free myostatin prodomain. This interaction is characterized by a fast kinetic profile, and has a dissociation constant of 1 μM. The WFIKKN1 binding site on the prodomain is only partially accessible in promyostatin.

4.3. WFIKKN1 precipitates the myostatin dimer through its prodomain from the latent myostatin preparations. The WFIKKN2 protein, which forms a very strong interaction with active myostatin, can access its binding site on the myostatin growth factor domain only after the BMP1 mediated cleavage of prodomains.

4.4. WFIKKN1 is a more potent inhibitor of the latent myostatin preparations than WFIKKN2.

5. The K153R mutation enhances promyostatin processing by the proprotein convertase furin.

5.1. K153R mutation has no effect on the activity of myostatin.

5.2. K153R mutation increases the rate of promyostatin cleavage by furin.
5.3. K153R mutation has no effect on the BMP1 mediated cleavage of prodomains in latent myostatin.

**Conclusion**

Based on the available data the WFIKKN2 protein is a specific and potent inhibitor of myostatin and GDF11 receptor binding.

WFIKKN1 and WFIKKN2 significantly differ in their myostatin inhibitor functions regarding the interaction with the myostatin prodomain. WFIKKN1 is capable of developing a biologically relevant interaction with the myostatin prodomain, whereas WFIKKN2 is not. According to my results the dissociated fraction of latent myostatin possess receptor binding activity. This activity could be attributable to the semilatent myostatin. The WFIKKN1 protein due to its prodomain binding capacity is a more potent inhibitor of the semilatent myostatin receptor binding activity, than WFIKKN2. According to published literature and our findings semilatent myostatin binding is part of the myostatin inhibition strategy of WFIKKN1, while WFIKKN2 is more effective in mature myostatin binding.

Furin processes K153R mutant promyostatin more efficiently than the wild type protein. Humans carrying the K153R myostatin mutation presumably have a higher concentration of active myostatin due to enhanced furin mediated promyostatin processing. Accordingly, humans carrying this mutation have weaker skeletal muscles and are prone to obesity.
Publications

Katalin Kondás, György Szláma, Mária Trexler, László Patthy: **Both WFIKKN1 and WFIKKN2 have high affinity for growth and differentiation factors 8 and 11.** *J. Biol. Chem.* 283, 23677–23684 (2008).

György Szláma, Katalin Kondás, Mária Trexler, László Patthy: **WFIKKN1 and WFIKKN2 bind growth factors TGFβ1, BMP2 and BMP4 but do not inhibit their signalling activity.** *FEBS J.* 277, 5040–5050 (2010).


György Szláma, Mária Trexler, László Patthy: **Latent myostatin has significant activity and this activity is controlled more efficiently by WFIKKN1 than by WFIKKN2.** *FEBS J.* 280, 3822–3839 (2013).