THE FUNCTIONAL CHARACTERIZATION OF THE HUMAN WFIKKN1 PROTEIN

Ph.D. thesis

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Budapest, 2011

Introduction

Using sensitive homology search and gene finding programs, the Functional Genomics Group of the Institute of Enzymology, BRC, HAS has identified two genomic regions on human chromosomes 16 and 17 which encode related extracellular multidomain proteins. Each protein contains a WAP, a follistatin, an immunoglobulin, two Kunitz-type and a netrin domain. Since WAP, follistatin and Kunitz domains frequently occur in various serine peptidase inhibitors, while the NTR domain often occurs in metallopeptidase inhibitors, it was assumed that WFIKKN proteins are multivalent peptidase inhibitors.

Recently, our group has shown that the second Kunitz-type domain of the WFIKKN1 protein is a trypsin-specific inhibitor, but based on the structural and evolutionary analyses, the hypothesis that the primary task of the KU2 domain would be the inhibition of trypsin was rejected.

Results published by J. Hill *et al.* in 2003 opened a new direction in the studies on the biological roles of WFIKKN proteins: the human WFIKKN2 protein was shown to inhibit the biological activity of GDF8 and GDF11 growth factors belonging to the TGFβ growth factor family.

Based on these results, it is assumed that the main function of the WFIKKN1 protein is similar to that of the paralogue WFIKKN2 protein: it may play a role in regulating the activity of some growth factors of the TGFβ family.

Objectives

The main objective of my thesis work was to identify the interacting partners of the WFIKKN1 protein within the TGFβ growth factor family, to characterize the interaction and to examine their biological importance.

Research plan:

1. Recombinant expression of various domain variants of the WFIKKN1 protein.
2. Analyse the interactions of the recombinant WFIKKN1 protein and TGFβ family members, and determine the kinetic parameters of these interactions.
3. Study the effect of the WFIKKN1 protein on binding of growth factors to their receptors using SPR solution competition assay. For these studies, expression of the extracellular domains of receptors had to be solved.
4. Study the effect of the WFIKKN1 protein on the biological activity of the growth factors using luciferase reporter gene assays.

Methods

The pPICZαA expression vectors containing the DNA fragment which encodes the KU2-NTR domain variant of WFIKKN1 and the extracellular domains of receptors were constructed using standard recombinant DNA technology protocols. *Pichia pastoris* strain GS115 was used to express recombinant proteins. These proteins were purified using Ni-affinity chromatography and gel filtration methods. The recombinant WFIKKN1 protein was expressed in *Drosophila melanogaster* S2 cells, the GDF8 prodomain was expressed in *Escherichia coli* BL21 cells, the WAP-FS, FS, KU1-KU2, NTR recombinant domain variants of WFIKKN1 were expressed in the *Pichia pastoris* GS115 strain by Mária Trexler. The growth factors were purchased from R&D Systems.

Depending on the size of the protein, 11-22% linear polyacrylamide gradient gels or 16% slab gels were used to analyze the composition of protein samples under both reducing and non-reducing conditions.

The molar extinction coefficients and the molecular weight of proteins were calculated using ProtParam, an online protein analysis application.

The concentration of purified recombinant proteins was determined using spectrophotometer.

Structural integrity of the monomeric recombinant proteins was verified using N-terminal sequence analysis on an Applied Biosystems 471A protein sequencer and using CD spectra measured with a JASCO J-720 spectropolarimeter. The melting temperature of the recombinant proteins was determined by derivative processing of changes in CD using the spectra analysis program for JASCO.

Protein-protein interactions were measured by surface plasmon resonance (SPR) on a BIAcore X instrument using CM5 sensor chips. Kinetic parameters for each interaction were determined by BIAevaluation software 4.0.

The ability of WFIKKN1 protein and its domain variants to block the binding of growth factors to their receptors was studied by SPR solution-competition assays.

The effect of WFIKKN1 protein on the activity of growth factors was measured by luciferase reporter gene assays. The GDF8, GDF11 activity assays were carried out on Rhabdomyosarcoma A204 cells transiently transfected with Cignal SMAD Firefly luciferase reporter vector and Renilla luciferase control vector, BMP2 and BMP4 activity assays were performed on HepG2-BRA cells stably transfected with the BRE-luc reporter vector, and TGFβ1 activity assays were carried out on MLEC-clone32 cells stably transfected with a PAI-1 promoter/Firefly luciferase vector. The luciferase activity was measured on an Appliskan luminometer.

Results

1. Characterization of the interaction between WFIKKN1 and GDF8, GDF11 proteins.

1.1 Surface Plasmon Resonance (SPR) studies have shown that WFIKKN1 binds to the GDF8 growth factor with high affinity, the calculated equilibrium dissociation constant is 3.35×10^{-8} M. On the domain level, the follistatin domain of WFIKKN1 protein (K_d: 2.71×10^{-7} M) binds with high affinity, and the netrin domain of WFIKKN protein (K_d: 2.38×10^{-6} M) binds with weaker affinity to the growth factor.

1.2 WFIKKN1 binds to the GDF8 prodomain as well (K_d: 4.85×10^{-7} M). This interaction is approximately fifteen times weaker than the interaction between GDF8 growth factor and WFIKKN1 protein. The NTR domain is responsible for this interaction (K_d: 1.6×10^{-7} M).

1.3 WFIKKN1 binds to the GDF8-related GDF11 growth factor with high affinity. The calculated equilibrium dissociation constant for the interaction between WFIKKN1 and GDF11 is 2.25×10^{-9} M.

2. Study on the biological importance of the interaction between WFIKKN1 and the GDF8, GDF11 proteins

2.1 GDF8 activates the signal transduction pathway through activin type II receptors (primarily to ACVRIIB). For the SPR solution-competition assays, the extracellular domain of ACVRIIB was expressed in *Pichia pastoris* expression system. Based on the solution-competition assay results, WFIKKN1 blocked the binding of growth factors to their receptors in a concentration-dependent manner. On the domain level, the netrin domain of WFIKKN1 has no role in the inhibition: inhibition is mediated by the follistatin domain.

2.2 WFIKKN1 efficiently inhibited the activity of GDF8 and GDF11 growth factors in the nanomolar concentration range in luciferase reporter gene assays: half maximal inhibition of 0.8 nM GDF8 is achieved by ~ 6 nM WFIKKN1 and 50 nM WFIKKN1 decreased the activity of 0.8 nM GDF11 to 20%.

3. Characterization of the interaction between WFIKKN1 and BMP2, BMP4, TGFβ1, activin A, BMP3 and BMP8b proteins

3.1 Based on SPR interaction analyses, WFIKKN1 binds to BMP2 (K_d: 7.25 x 10^{-7} M), BMP4 (K_d: 8.2 x 10^{-7} M) and TGFβ1 (K_{d1}: 4.5 x 10^{-7} M; K_{d2}: 8.93 x 10^{-5}) growth factors with approximately the same affinity, although the affinities of WFIKKN1 for these growth factors are significantly lower than those determined for GDF8 and GDF11. WFIKKN1 binds to BMP3 (K_d: 3.37 x 10^{-6} M) and BMP8b (K_d: 3.06 x 10^{-5} M) proteins with very low affinities only. No interaction was detected with activin A.

4. Study on the biological importance of the interactions between WFIKKN1 and BMP2, BMP4, TGFβ1 proteins

4.1 BMP2 and BMP4 growth factors bind to BMP type I receptors. For the SPR solution-competition assays, the extracellular domain of BMPRIA was expressed in *Pichia pastoris* expression system. Based on the SPR solution-competition assay results, WFIKKN1 blocks the binding of BMP2 and BMP4 to their receptors very weakly.

4.2 WFIKKN1 has no effect on the activity of BMP2, BMP4 and TGFβ1 growth factors even in the micromolar range in luciferase reporter gene assays.

Conclusions

Similarly to human WFIKKN2 protein, the human WFIKKN1 protein specifically inhibits the activity of GDF8 and GDF11 and may thus play a significant role in the regulation of the biological processes controlled by these growth factors.

In the case of BMP2, BMP4 and TGFβ1 growth factors, WFIKKN1 may function as a growth factor binding protein. It is suggested that physical association of WFIKKN1 with these growth factors may localize their action and may thus help to establish growth factor gradients in the extracellular space.

My results suggest that WFIKKN1 and WFIKKN2 proteins show remarkable similarities: both proteins specifically inhibit the activity of GDF8 and GDF11, and neither have an effect on the activities of activin A and TGFβ1 growth factors in luciferase reporter gene assays. Since the expression patterns of human WFIKKN proteins show significant differences, it is possible that functional differences between the proteins occurs at the level of expression pattern rather than at the level of ligand-specificity. In other words, it is the expression pattern of WFIKKN1 and WFIKKN2 that determines which of them may be responsible for the control of the activity of GDF8 or GDF11 in a given tissue.

Publications

Publications discussed in the Ph.D. work:

Kondás K., Szláma G., Trexler M. and Patthy L. (2008) Both WFIKKN1 and WFIKKN2 have high affinity for Growth and Differentiation Factors 8 and 11. *J. Biol. Chem.* **283**, 23677-23684.

Szláma G., Kondás K., Trexler M. and Patthy L. (2010) WFIKKN1 and WFIKKN2 bind growth factors TGFβ1, BMP2 and BMP4 but do not inhibit their signalling activity. *FEBS J.* **277**, 5040-5050.