FEBS Letters 583 (2009) 19–24

FEBS Letters

journal homepage: www.FEBSLetters.org

FGF21 N- and C-termini play different roles in receptor interaction and activation

Junming Yie [a,*], Randy Hecht [b], Jennifer Patel [a], Jennitte Stevens [b], Wei Wang [b], Nessa Hawkins [b], Shirley Steavenson [b], Steve Smith [b], Dwight Winters [b], Seth Fisher [b], Ling Cai [b], Ed Belouski [b], Ching Chen [b], Mark L. Michaels [b], Yue-Sheng Li [b], Richard Lindberg [a], Minghan Wang [a], Murielle Véniant [a], Jing Xu [a]

[a] Department of Metabolic Disorders, Amgen Inc., One Amgen Center Drive, Mail Stop 29-1-A, Thousand Oaks, CA 91320, USA
[b] Department of Protein Science, Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA 91320, USA

ARTICLE INFO

Article history:
Received 11 September 2008
Revised 20 October 2008
Accepted 9 November 2008
Available online 4 December 2008

Edited by Gianni Cesareni

Keywords:
Fibroblast growth factor-21
β-Klotho
Fibroblast growth factor receptor
Partial agonist

ABSTRACT

Fibroblast growth factor-21 (FGF21) signaling requires the presence of β-Klotho, a co-receptor with a very short cytoplasmic domain. Here we show that FGF21 binds directly to β-Klotho through its C-terminus. Serial C-terminal truncations of FGF21 weakened or even abrogated its interaction with β-Klotho in a Biacore assay, and led to gradual loss of potency in a luciferase reporter assay but with little effect on maximal response. In contrast, serial N-terminal truncations of FGF21 had no impact on β-Klotho binding. Interestingly, several of them exhibited characteristics of partial agonists with minimal effects on potency. These data demonstrate that the C-terminus of FGF21 is critical for binding to β-Klotho and the N-terminus is critical for fibroblast growth factor receptor (FGFR) activation.

Structured summary:
MINT-6799939: *FGFR1c* (uniprotkb:P11362) *binds* (MI:0407) to *β-Klotho* (uniprotkb: Q86Z14) by *surface plasmon resonance* (MI:0107)
MINT-6799907, MINT-6799922: *FGF21* (uniprotkb: Q9NSA1) *binds* (MI:0407) to *β-Klotho* (uniprotkb: Q86Z14) by *surface plasmon resonance* (MI:0107)

1. Introduction

Fibroblast growth factor-21 (FGF21) belongs to the FGF19 subfamily, which consists of FGF19, 21 and 23 [1]. The FGF19 subfamily members have emerged as potent endocrine hormones involved in the regulation of diverse physiological homeostasis [2–4]. Administration of recombinant FGF21 lowered plasma glucose and insulin levels, reduced hepatic and circulating triglycerides and cholesterol levels, and improved insulin sensitivity, energy expenditure, hepatic steatosis and obesity in a range of insulin resistant animal models [4–6]. Hence, FGF21 has become an attractive therapeutic agent to treat human type 2 diabetes and the associated metabolic syndrome.

Recent progress implied that functional differences between FGF21 and traditional FGFs may arise from differences in their receptor signaling components [7]. Traditional FGFs signal through a family of high affinity receptor tyrosine kinases (FGFR) in the presence of heparin sulfate proteoglycan (HSPG), a low affinity co-receptor required for FGF/FGFR interactions (see review [8] and references therein). In contrast, the FGF19 subfamily requires distinct Klotho transmembrane proteins as co-receptor for the activation of FGFR signaling [9]. α-Klotho is required for FGF23 to regulate renal phosphate metabolism [10], whereas β-Klotho is required for FGF21 to regulate glucose and lipid metabolism [11] and FGF19 to regulate bile acid metabolism [12] FGF21 is active in adipocytes and pancreatic β-cells, which express both FGFRs and β-Klotho [4,13,14]. Interestingly, when β-Klotho was transfected in non-responding fibroblasts, FGF21-induced signaling was observed [11,15]. In addition, immunoprecipitation studies indicate that β-Klotho interacts with FGF21 and facilitates the formation of the FGF21–β-Klotho–FGFR ternary complex [11,15,16]. Moreover, siRNA knockdown of β-Klotho abolished FGF21-induced signaling and its stimulatory effect on GLUT1 expression and glucose uptake in 3T3-L1 adipocytes [16].

The FGFR isoforms mediating FGF21 signaling are not completely defined. It is known that FGFR4 and the c-isoforms of FGFR1, 2 and 3 are possible candidates [11,15–17]. Immunoprecipitation experiments showed that β-Klotho interacts with FGFR1c and FGFR4 more efficiently than FGFR2c and 3c [11,15,16]. However, in the presence of β-Klotho, FGF21 could signal in BaF3 cells expressing either FGFR1c or 3c, but not 2c or FGFR4 [18]

Abbreviations: FGF, fibroblast growth factor; ERK, extracellular signal-regulated kinase; HSPG, heparin sulfate proteoglycan

* Corresponding author. Fax: +1 805 499 0953.
E-mail address: jyie@amgen.com (J. Yie).

0014-5793/$34.00 © 2008 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2008.11.023

and in L6 myoblasts expressing FGFR1c, 2c or 3c, but not FGFR4 [16]. Therefore, the c-isoforms of FGFRs rather than FGFR4 might be physiologically relevant receptors for FGF21 and mediate FGF21 activity through their respective tissue expression patterns. Independently, FGFR4 has been shown to be a specific receptor for FGF19 [19].

Despite the progress made in the identification of FGF21 receptor signaling components, the nature of the interactions between FGF21, FGFR and β-Klotho is not completely understood. Structure-based modeling and sequence alignment suggest that FGF21 has a typical 120 aa β-trefoil core structure like the traditional FGFs, but its N- and C-terminal sequences are significantly different from those of other FGFs ([9], and unpublished data). Here we sought to use FGF21 N- and C-terminal truncations to identify the regions of FGF21 responsible for FGFR and β-Klotho interactions and thereby better understand these regions' roles in FGF21 receptor activation.

2. Materials and methods

2.1. FGF21, β-Klotho and FGFR1c protein and plasmids

Full-length and truncated human FGF21 proteins were expressed in an *Escherichia coli* strain using a proprietary vector. The expression was primarily found in inclusion bodies. The recovery of oxidized proteins involved the dissolution of the inclusion bodies and refolding of the soluble reduced protein. Briefly, *E. coli* cells were resuspended in water and lysed with a 110S Microfluidizer. The inclusion bodies were dissolved with a buffer of 8 M guanidine, 50 mM Tris, pH 8.5, and 10 mM dithiothreitol. The solution was subsequently added to a refolding solution to achieve a final concentration of 1 mg/ml. The refolded material was further purified by anion exchange chromatography, followed by hydrophobic interaction chromatography. All deletion mutants were tested by Western blotting and reverse-phase HPLC to ensure protein integrity. No deletion resulted in significant structural changes as judged by spectrum photometric measurement.

The extracellular domain of human β-Klotho (1–992) fused with 6xHis tag at the C-terminus was cloned into the pTT14 expression vector. The extracellular domain of β-Klotho protein was purified from a 293F stable cell line using Talon IMAC resin-Co^{2+} (Clontech) according to the manufacturer's protocol, and was further purified with CHT Type I 40 μm column (Bio-Rad). The recombinant FGFR1c-Fc fusion protein with the extracellular domain of FGFR1c at the N-terminus was generated internally with similar method as the protein available commercially from R&D systems.

2.2. ELK luciferase assay

ELK luciferase assay was performed in 293T cells that were stably transfected with human β-Klotho and reporter constructs containing 5xUAS luciferase and GAL4 DNA-binding domain (DBD) fused to ELK1. In this system, luciferase activity is regulated by the endogenous phosphorylated extracellular signal-regulated kinase (ERK). The 293T stable cells were seeded at 1×10^5 cells/well on 96-well plates. On the next day, FGF21 proteins were added to the media. The plates were incubated for 6 h. Cells were then lysed to measure luciferase activity using the Bright-Glo luciferase assay system (Promega).

2.3. Biacore binding studies

Binding of FGF21 and its mutants to human β-Klotho was tested in a Biacore solution equilibrium binding assay. Briefly, NeutrAvidin (Pierce) was immobilized on a CM5 chip using amine coupling reagents (GE Healthcare). Biotinylated FGF21 was captured on the second flow cell to 800RU. The biotinylated FGF21 has similar activity as native FGF21 in the luciferase assay and the Biacore binding assay (data not shown). Each FGF21 mutant was incubated at three different concentrations (2 μM, 200 nM and 20 nM) with 10 nM human β-Klotho in a PBS buffer containing 0.1 mg/ml BSA and 0.005% P20 for 1 h at room temperature. The pre-incubated mixtures were injected over the biotin–FGF21 surface to measure the binding of free β-Klotho. The signal obtained with no FGF21 mutant in the pre-incubation represents 100% β-Klotho binding and serves as control. Decreased signal with increasing concentrations of a given FGF21 mutant in the pre-incubation indicates that it blocks β-Klotho binding to the immobilized biotin–FGF21 and therefore, retains ability to bind to β-Klotho.

Binding of human β-Klotho to FGFR1c-Fc was also tested in the Biacore assay. Briefly, FGFR1c-Fc was immobilized on the second flow cell of a CM5 chip using amine coupling to ~10000 RU. The first flow cell was used as a background control. Twenty nanomolars of human β-Klotho diluted in PBS plus 0.1 mg/ml BSA and 0.005% surfactant P20 was injected over the FGFR1c-Fc surface for 5 min at 10 μl/min.

2.4. Statistical analysis

All data are means ± standard deviation (S.D.). Values were compared using Student's paired *t*-test. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Direct interaction of FGF21 with β-Klotho

We developed a luciferase reporter assay system in 293T cells that stably express human β-Klotho and reporter constructs. Then, we measured and determined that these cells express multiple FGFRs including FGFR1c endogenously (data not shown). When FGF21 was tested in this cell line, ERK phosphorylation was induced leading to elevated luciferase activity (EC_{50} = 0.5 nM, Fig. 1A). To test whether FGF21 could directly bind to β-Klotho or FGFR, we set up a competition assay using the same reporter system. FGFR1c was selected as the FGFR since it is the most validated receptor for the FGF21 signaling [11,15–17]. Either soluble β-Klotho alone or FGFR1c-Fc alone did not affect luciferase activity (data not shown). However, soluble β-Klotho decreased FGF21-induced luciferase activity in a dose-dependent manner ($IC_{50} \sim 110$ nM, Fig. 1B), suggesting that the soluble β-Klotho may directly bind to FGF21 and sequester it from acting on the cells. In contrast, FGFR1c-Fc did not inhibit FGF21 signaling (Fig. 1C), indicating that no interaction between FGFR1c and FGF21 may have occurred. Interaction between β-Klotho and FGF21 was further assessed in a direct binding study using Biacore. Interaction between FGF21 and β-Klotho was detected (Fig. 1D). Soluble FGF21 inhibited β-Klotho binding to immobilized FGF21 with an IC_{50} around 18.6 nM (Fig. 1D). The estimated Kd (k_{off}/k_{on}) value is approximately 15 nM. No direct binding between FGFR1c and FGF21 was detected (data not shown), a result that is consistent with the finding from the luciferase assay where soluble FGFR1c did not inhibit FGF21 signaling (Fig. 1B). Other FGFRs such as FGFR2c, FGFR3c and FGFR4 were also tested in the Biacore assay and no direct binding between these FGFRs and FGF21 was detected (data not shown).

3.2. Synergism of FGFR1c and β-Klotho in FGF21 signaling

Several immunoprecipitation experiments have shown that β-Klotho interacts with FGFR1c [11,15,16]. Surprisingly, β-Klotho/