Regulation of Fibroblast Growth Factor-23 Signaling by Klotho

The aging suppressor gene Klotho encodes a single-pass transmembrane protein. Klotho-deficient mice exhibit a variety of aging-like phenotypes, many of which are similar to those observed in fibroblast growth factor-23 (FGF23)-deficient mice. To test the possibility that Klotho and FGF23 may function in a common signal transduction pathway(s), we investigated whether Klotho is involved in FGF signaling. Here we show that Klotho protein directly binds to multiple FGF receptors (FGFRs). The Klotho-FGFR complex binds to FGF23 with higher affinity than FGF or Klotho alone. In addition, Klotho significantly enhanced the ability of FGF23 to induce phosphorylation of FGF receptor substrate and ERK in various types of cells. Thus, Klotho functions as a cofactor necessary for activation of FGF signaling by FGF23.

Materials and Methods

Expression Vectors—Complementary DNA containing the mouse FGFRs coding region (IMAGE Clone, Invitrogen, supplemental Fig. 1) were cloned into pcDNA3.1(+) expression vector (Invitrogen). Before subcloning, a V5-epitope tag was added to the C terminus and appropriate restriction enzyme sites to the both ends using synthetic oligonucleotides and polymerase chain reaction. Expression vectors for the mouse FGF23 resistant to proteolytic inactivation (R179Q) (18), the transmembrane form of mouse Klotho, and the extracellular domain of mouse Klotho were cloned into pcDNA3.1(+) (Invitrogen) in the same way except that a FLAG-epitope tag was added to the C terminus.

Cell Culture and Transfection—All cells except PC12 cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. PC12 cells were maintained in the same medium with additional 10% horse serum. Stable transformants of 293 cells expressing the full-length transmembrane form of Klotho (293KL) or the extracellular domain of Klotho (293KL ΔTM) were isolated after selection with G418 (Invitrogen) for 14 days. Subconfluent 293, 293KL, and 293KL ΔTM cells were transfected with the FGF expression vector plasmids using the Lipofectamine transfection reagent (Invitrogen) according to the manufacturer’s protocol. A CHO cell line that stably expresses the full-length Klotho (CHOKL) is a gift from Kyowa Hakko Kogyo Co. Ltd. (5).

Adenovirus Construction—A CDNA encoding the full-length transmembrane form of mouse Klotho with a C-terminal myc-epitope tag or green fluorescent protein (GFP) was inserted into the multiple cloning site of pShuttle-CMV (Qiagen). After linearization, the shuttle vector was introduced into an electroporation-competent Escherichia coli B5183-AD-1 harboring the adenoviral backbone pΔEasy-1 (Strategene). The recombinant vector was introduced into the adenovirus packaging cell line QBI-HEK293A (Qibogene) using FuGene 6 (Roche Applied Science). The viruses were amplified by several rounds of infection in QBI-HEK293A cells. Subconfluent HeLa cells or PC12 cells were infected with the adenovirus expressing Klotho or GFP (m.o.i. = 3 for HeLa and m.o.i. = 10 for PC12) 36 h before stimulation with FGF23 and then subjected to immunoblot analysis of FGF signaling pathway as described below.

Immunoprecipitation and Immunoblotting—To prepare cell lysate, cells were snap-frozen in liquid nitrogen and lysed in the lysis buffer containing inhibitors for phosphatase and proteinase as described previously (2). The lysis of 293KL or 293KL ΔTM cells transfected with expression vectors for FGFRs was incubated with agarse beads conjugated with anti-V5 antibody (Sigma) or anti-FLAG antibody (Sigma) at 4 °C for 3 h. The beads were washed three times with Tris-buffered saline (TBS) containing 1% Triton X-100 (TBST) and three times with TBS. The washed beads were suspended in SDS-sample loading buffer and subjected to SDS-PAGE. The protein transferred to Hybond C Extra membrane (Amersham Biosciences) was incubated with anti-Klotho rat monoclonal antibody KM2119 (19) or anti-V5 antibody (Invitrogen) and then with horseradish peroxidase-linked secondary antibodies (Amersham Biosciences). The signals were detected with Supersignal West Dura system (Pierce). For detecting Klotho binding to endogenous FGFRs in 293KL cells, cell lysate was immunoprecipitated with anti-FLAG-agarose in the same way as described above and then immunoblotted with antibodies against FGFR1 (Santa Cruz Biotechnology), FGFR2 (Santa Cruz Biotechnology), FGFR3 (Sigma), or KM2119.

Preparation of Conditioned Medium Containing FGF23 (R179Q)—Serum-free conditioned medium was prepared by transfecting 293 cells with the
Co-precipitation of Endogenous Klotho and FGFRs from Mouse Kidney—Kidney from a 129 mouse (200 mg) was homogenized in 2 ml of homogenizing buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 0.5 mM EDTA) containing protease inhibitors. The homogenate was incubated for 30 min at 4 °C after the addition of Triton X-100 (final 1%) and then centrifuged for 12 min at 18,000 × g two times to remove debris. The supernatant was preclearred with 40 μl of protein A-Sepharose (Amersham Biosciences) conjugated with 20 μg of normal rabbit IgG for 2.5 h at 4 °C. The precleared lysate was incubated with 20 μg of protein A-Sepharose conjugated with 16 μg of anti-FGFR1 antibody or normal rabbit IgG for 2.5 h at 4 °C. The beads were processed in the same way as described above for immunoblot analysis using KM2119 and anti-FGFR1 antibody. The lysate of the kidney immunoprecipitated with anti-Klotho antibody KL11-A (Alpha Diagnostic International) was used as a positive control for Klotho. The mouse FGFR1c with a V5-epitope tag expressed in 293 cells was used as a positive control for FGFR1.

**FGF23 Pull-down Experiments**—Lysate of 293 cells or 293KL cells transfected with the FGFR expression vectors was applied to anti-V5-agarose at 4 °C for 3 h. Serum-free conditioned medium of 293 cells or 293KLΔTM cells was applied to anti-FLAG-agarose at 4 °C for 3 h. The beads were washed four times with TBST and then incubated with conditioned medium of 293 cells transfected with the mouse FGF23 (R179Q) expression vector supplemented with 0.5% Triton X-100 at 4 °C for 3 h. The beads were washed three times with Krebs-Ringer-HEPES buffer containing 1% Triton X-100 and then three times with the same buffer without Triton X-100. The washed beads were suspended in SDS-sample loading buffer and subjected to immunoblot analysis using anti-V5 antibody, KM2119, or anti-FGFR2 antibody (R&D Systems).

**Immunoblot Analysis of the FGF Signaling Pathway**—Subconfluent 293, 293KL, and 293KLΔTM cells grown on 6-well plates were serum-starved overnight and then treated with various concentrations of recombinant human FGF23 (0, 0.1, 1, 10, or 100 ng/ml, Genzyme), acidic or basic FGF (Upstate) for 15 min. Conditioned medium of 293 cells transfected with the expression vector for mouse FGF23 (R179Q) was also used as a source of FGF23 where indicated. The cell lysates were subjected to immunoblot analysis using anti-phospho-FRS2α antibody (Cell Signaling), anti-phospho-p44/42 MAP kinase (ERK1/2) antibody (Cell Signaling), or anti-ERK antibody (Cell Signaling).

**RESULTS**

To test the hypothesis that Klotho may be involved in FGF signaling, we investigated whether Klotho could directly bind to FGFRs. Four receptor tyrosine kinases are designated as the high affinity FGFRs (FGFR1–4). Alternative RNA splicing generates multiple FGFR isoforms. The extracellular domain of FGFR1 and FGFR2 is composed of either two or three immunoglobulin-like (Ig-like) ligand-binding domains. Alternative splicing in the third Ig-like domain exists in FGFR1–3, generating “b” and “c” isoforms (20). We transfected 293KL cells that stably expressed the full-length transmembrane form of Klotho with various FGFR expression vectors and asked if any of the FGFRs would be immunoprecipitated with Klotho. Klotho bound to almost all FGFR isoforms tested (Fig. 1). However, significant difference in the ability of Klotho to co-precipitate FGFRs was observed between the isoforms: 1) c isoforms were more efficiently co-precipitated with Klotho than b isoforms. The difference between b and c isoforms is located in the C-terminal half of the third Ig-like ligand-binding domain and known to affect binding affinity to FGFs as well (21). 2) FGFR2 was less efficiently co-precipitated with Klotho than FGFR1, FGFR3, and FGFR4. Thus, Klotho may bind to multiple FGFRs with different affinity.

To test whether binding of Klotho to FGFRs may affect interaction between FGFRs and FGF23, the ability of FGFRs to pull down FGF23 was examined in the presence and absence of Klotho. FGF23 was pulled down with FGFR1c, -3c, and -4 only in the presence of Klotho (Fig. 2). We tested the possibility that FGF23 might directly bind to Klotho. However, the extracellular domain of Klotho alone failed to pull down FGF23 under these experimental conditions (Fig. 2). These observations indicate that FGF23 show stronger interaction with the Klotho-FGFR complex than with Klotho or FGFR alone.

Since Klotho increased binding of FGF23 to FGFRs, it might enhance the ability of FGF23 to activate FGF signaling. To test this possibility, we stimulated 293KL cells or 293 cells with various concentration of recombinant human FGF23 and compared phosphorylation of FGF receptor substrate-2α (FRS2α) and p44/42 MAP kinase (ERK1/2) in 293KL cells (Fig. 3A), indicating that Klotho enhanced the cellular sensitivity to FGF23 >10 times without the help of heparin or glycosaminoglycan. The similar results were obtained using two independent 293KL clones (data not shown). In addition,
we observed FGF23-induced FRS2α and ERK phosphorylation in 293KLΔTM cells (Fig. 3B), although the levels were reduced relative to those in 293KL cells. Consistent with these findings, endogenous FGFRs in 293KL cells were co-precipitated with the full-length transmembrane Klotho and, to a smaller extent, with the extracellular domain of Klotho (Fig. 3C). We also confirmed that the extracellular domain of Klotho bound to exogenously expressed FGFRs in the same pattern as the full-length Klotho did (supplemental Fig. 2). Based on these observations, we conclude that both the full-length Klotho and the extracellular domain of Klotho function as cofactors necessary for efficient activation of FGF signaling by FGF23 (Fig. 3D).

The primary function of FGF23 is to suppress phosphate reabsorption in the renal tubular cells. Indeed, interaction between endogenous Klotho and FGFR was observed in the mouse kidney (supplemental Fig. 3). To test whether the activity of Klotho to enhance FGF23 action might be observed in non-kidney cells as well, we infected PC12 and HeLa cells, which originated from pheochromocytoma and cervical carcinoma, respectively, with adenovirus expressing the full-length Klotho and then stimulated with FGF23. In addition, we stimulated stable transformants of ovary-derived CHO cells expressing the full-length Klotho with FGF23. These non-kidney cells acquired the ability to respond to FGF23 when Klotho was expressed (Fig. 4).

Since Klotho binds to multiple FGFRs, it may affect the activity of FGFs other than FGF23. We stimulated 293 or 293KL cells with various doses of acidic and basic FGF and found that Klotho did not enhance their ability to activate FGF signaling but slightly suppressed basic FGF action (Fig. 3A and supplemental Fig. 4).

**DISCUSSION**

The fact that FGF23 requires Klotho to activate FGF signaling may explain why Klotho−/− mice develop all the phenotypes observed in Fgf23−/− mice. However, Klotho−/− mice show many phenotypes not described in Fgf23−/− mice, including arteriosclerosis, ectopic calcification in extra-renal tissues, skin atrophy, neuronal degeneration, and pulmonary emphysema (1). This may imply that Klotho affects the activity of multiple FGFs through binding to multiple FGFRs, although the effect of Klotho on acidic and basic FGF was small when compared with its robust effect on FGF23 (supplemental Fig. 4). Precise comparison between Klotho−/− and Fgf23−/− phenotypes may provide a clue to understanding of potential effects of Klotho on the other FGFs besides FGF23.

In the absence of Klotho, FGF23 requires exogenous heparin or glycosaminoglycan as a cofactor to stimulate FGF signaling (13, 15, 16), indicating that the help of sugar chains are critical for the biological activity of FGF23. Klotho is a glycoprotein and detected as two bands by immunoblot analysis (Fig. 1), which represent different glycosylation (5). We noticed that the upper band of Klotho was enriched when Klotho was co-precipitated with the high affinity binding partners such as FGFR1c, -3c, and -4 (Fig. 1). It is possible that a particular sugar chain(s) on Klotho protein may be involved in the high affinity interaction between FGFRs and FGF23. Furthermore, binding of FGFRs to the upper band of Klotho was associated with the ability of the Klotho-FGFR complex to pull down FGF23 (Fig. 3).

The fact that the extracellular domain of Klotho can increase cellular sensitivity to FGF23 (Fig. 3B) has raised the possibility that it may function as a paracrine factor in the kidney. Since Klotho is expressed in the distal convoluted tubules (1) and the extracellular domain of Klotho is shed and secreted (2, 5), it may act on adjacent proximal tubules and work cooperatively with FGF23 to inhibit phosphate reabsorption. It remains to be determined whether extra-cellular Klotho peptide could function as a paracrine and/or an endocrine factor in the regulation of FGF23 signaling.

It was recently reported that mice defective in βKlotho, a protein that structurally resembles Klotho, showed increased synthesis and excretion of bile acids (22). Interestingly, these phenotypes are identical with those observed in Fgf4 knockout mice (23, 24). The Klotho gene family may have evolved in primates to provide better understanding of the complex FGF signaling system and its relation to aging.

**REFERENCES**

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