FGF19 is a unique member of the FGF family of secreted proteins that regulates bile acid homeostasis and metabolic state in an endocrine fashion. Here we investigate the cell surface receptors required for signaling by FGF19. We show that βKlotho, a single-pass transmembrane protein highly expressed in liver and fat, induced ERK1/2 phosphorylation in response to FGF19 treatment and significantly increased the interactions between FGF19 and FGFR4. Interestingly, our results show that αKlotho, another Klotho family protein related to βKlotho, also induced ERK1/2 phosphorylation in response to FGF19 treatment, and increased FGF19/FGFR4 interactions in vitro similar to βKlotho effects. In addition, heparin further enhanced the effects of both α and βKlotho in FGF19 signaling and interaction experiments. These results suggest that a functional FGF19 receptor may consist FGFR, heparan sulfate complexed with either α or βKlotho.

The fibroblast growth factors (FGFs) constitute a structurally related family of 22 proteins (1). This family of secreted proteins has been implicated in a variety of functions including angiogenesis, mitogenesis, vertebrate and invertebrate development, cellular differentiation, wound healing/repair, and metabolic regulations (2, 3). FGFs can be grouped into seven subfamilies based on their sequence similarities and functional properties (4). Four tyrosine kinase receptors have been identified for FGFs (FGFR1-4), each containing an extracellular ligand binding domain, a single transmembrane domain, and an intracellular tyrosine kinase domain (5). Alternative RNA splicing of one of two unique exons in FGFR1-3 results in the two different, b and c, receptor isoforms (2). Because most FGFs only function in an autocrine or paracrine fashion, the tissue distribution of these FGFs determines the tissue specific functions for most of the FGF family members (2,4).

The FGF19 subfamily contains 3 members, FGF19, FGF21, and FGF23. In contrast to other FGFs which require heparin or heparan sulfate for high affinity receptor binding and activation, FGF19 subfamily members do not bind heparin with high affinity (6). In addition, FGF19 subfamily members contain intramolecular disulfide bonds which may function to increase their stability in plasma and allow them to function as hormones (7). Indeed, even though FGF19 is not expressed in liver or gallbladder, it can regulate hepatic bile acid metabolism and control gallbladder filling (8, 9, 10, 11). Furthermore, transgenic animals over-expressing FGF19 from skeletal muscle and animals injected with recombinant protein display improved insulin sensitivity, reduce adiposity, and increased metabolic rate (12,13). In addition, FGF21 has been shown to regulate glucose and lipid metabolism in an endocrine fashion (14), and FGF23 may function as a phosphaturic hormone (15). These unique features of the FGF19 subfamily suggest that they may interact with their receptors differently from the canonical FGFs and may require different receptor complexes to function.

The unique receptor requirements were first noted from studies with FGF23 (16,17). The αKlotho gene encodes a 130-kDa single-pass transmembrane protein with a short cytoplasmic domain. Since FGF23 deficient mice (FGF23/-mice) and αKlotho-deficient mice (αKlotho/-mice) share remarkable similarity in phenotypes,
including shortened life span, growth retardation, infertility, muscle atrophy, hypoglycemia, and vascular calcification in the kidneys (18,19), it was hypothesized that FGF23 and αKlotho function through a common signal transduction pathway. Direct evidence for αKlotho as a co-receptor for FGF23 came from recent biochemical and cellular studies. It was shown that although FGF23 alone has poor affinity for FGFRs and did not promote efficient activation of FGFRs in cells, αKlotho was able to function as an essential cofactor for the activation of FGFR signaling by FGF23. This suggests FGF23 binds an αKlotho-FGFR complex with higher affinity than to either receptor alone. Coexpression of αKlotho in 293 and CHO cells significantly enhanced the ability of FGF23 to induce phosphorylation of FGFR substrate and ERK1/2 in these cells (16, 17). Similar observations have recently been reported for FGF21. Instead of requiring αKlotho as a cofactor, FGF21 utilizes another Klotho family protein, βKlotho, as a coreceptor for signaling (20). Therefore, the presence of βKlotho confers high affinity binding of FGF21 to FGFRs and allows cells coexpressing βKlotho and FGFRs to respond to FGF21 and activate signal transduction pathways.

Though earlier observations suggest that heparin has low affinity interaction with FGF19 and may act as a weak cofactor for FGF19 (21), whether additional factors were required or could modulate FGF19-FGFR interactions and the effect of heparin on receptor complex formation have not been defined. Here, we show that both αKlotho and βKlotho may function as coreceptors for FGF19. The presence of αKlotho or βKlotho together with relatively high concentrations of heparin confers the strongest binding of FGF19 to FGFR, and results in the highest level of ERK1/2 phosphorylation following receptor activation.

**Experimental Procedures**

**Plasmids and Proteins.** Full length human βKlotho, αKlotho, extracellular domain of human βKlotho and αKlotho were cloned into the pTT14 expression vector. pFA2-Elk1 and pFR-Luc plasmids used in the Erk luciferase reporter assay were from Stratagene. Renilla luciferase, pRL-TK (Promega), was used as internal control reporter. Recombinant FGF19 and FGFR-Fc fusion proteins were purchased from R&D.

**Cell Culture and Transfections.** HEK293 cells were maintained in Dulbecco's modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were transfected with the expression vector plasmids using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol.

**Conditioned Medium Preparation.** HEK293 cells were transfected with expression vectors encoding the extracellular domains of βKlotho and αKlotho and the medium was collected three days after transfection. Expression of soluble β and αKlotho was confirmed by western blot using anti-β and αKlotho antibodies (R&D). Medium from cells transfected with vector encoding green fluorescent protein (GFP) was also collected as a negative control.

**Pull-down Assay.** To analyze the interaction between FGF19 and FGFRs, recombinant FGF19 (0.5 μg) was mixed with FGFR1c, FGFR2c, FGFR3c or FGFR4-Fc fusion proteins (0.5 μg) in conditioned medium and applied to 50 μl Protein G sepharose at 4 ºC for 2 hours. The beads were washed three times with PBS and then suspended in SDS sample buffer and subjected to western-blot analysis with anti-FGF19 antibody (R&D).

**ERK Activation Reporter Assay.** HEK 293 cells were seeded in 96-well plates (10^5 cells per well). For each well the transfection was done by mixing 10 ng pFA2-Elk1, 150 ng pFR-Luc and 10 ng pRL-TK reporter plasmids and 150 ng βKlotho expression vector in 25 μl OptiMEM (Invitrogen) with 0.5 μl Lipofectamine 2000 in 25 μl OptiMEM. On the following day, the cells were cultured in DMEM without serum but with 0.2% BSA overnight. The cells were incubated with various concentrations of FGF19 for 5 hours, and the luciferase activity was measured using DualGlo (Promega), according to the manufacturer’s instructions.
Western-blot Analysis of the FGF Signaling Pathway. HEK293 cells were transfected in 6-well plates and starved in serum-free medium overnight the day after transfection. For heparitinase treatment, 0.2 U/ml heparitinase (Sigma) was incubated with the cells for three hours before FGF19 was added. After treatment with various concentrations of recombinant human FGF19 for 15 min, cells were snap-frozen in liquid nitrogen and cell lysates were prepared in SDS sample buffer and subjected to western blot analysis using anti-phospho-p44/42 MAP kinase (anti-phospho-ERK1/2) antibody (Cell Signaling) and anti-ERK antibody (Cell Signaling).

RESULTS AND DISCUSSIONS

βKlotho and Heparin Enhance FGF19 Activation of FGFR Signaling. Since βKlotho is highly expressed in the liver and mice deficient in βKlotho exhibit a dramatic elevation of bile acid synthesis, we tested the possibility that βKlotho might function as a co-receptor for FGF19. We used a luciferase reporter assay system based on ERK1/2 phosphorylation (22) to test the activation of FGF signaling pathway by FGF19 in HEK293 cells which normally do not respond to FGF19 treatment. In HEK293 cells transfected with only control GFP vector, FGF19 did not activate any luciferase reporter expression (Fig 1). To test whether βKlotho is required for FGF19 activation of FGFR signaling, we transfected HEK293 cells with full length human βKlotho and stimulated the cells with various concentrations of FGF19. As shown in Fig. 1, βKlotho expressing cells responded to FGF19 in a dose dependent manner with an EC50 value of 54 nM. Although FGF19 has been reported to have significantly lower heparin binding affinity (6), adding heparin at a relatively high concentration of 20 μg/ml to the cells further enhanced the FGF19 response (Fig. 1). Heparin alone at this concentration had no effect on the reporter expression (Fig. 1). These results suggest that FGF19 may signal through the complex formed by βKlotho, heparin and FGFR receptors.

βKlotho and Heparin Enhance the Binding of FGF19 to FGFR4. The mammalian FGFRs are encoded by four distinct genes (FGFR1 through FGFR4). A major alternative mRNA splicing event within one of the three immunoglobulin-like extracellular domains of FGFR1-3 generates the “b” and “c” isoforms. It has been shown that both βKlotho and αKlotho preferentially bind to the c isoforms of FGFR1-3 and FGFR4 (17, 20). FGF21 interacted with FGFR1c, 2c, and 4 complexed with βKlotho (20), and FGF23 interacted with FGFR1c, 3c, and 4 complexed with αKlotho (17). To investigate the effects of βKlotho and heparin on the binding of FGF19 to the various FGFRs, we used pull-down experiments to test if FGF19 can be co-precipitated by Fc-FGFR fusion proteins complexed to protein G beads. In the presence of 20 μg/ml heparin, we demonstrated the binding of FGF19 to FGFR4, but did not detect significant binding to FGFR1c, 2c, and 3c (Fig. 2A left panel). When conditioned media containing the extracellular domain of βKlotho was added to the immunoprecipitation reaction, the FGF19-FGFR4 interaction was increased, but still no significant interactions could be observed between FGF19 and the other FGFRs (Fig. 2A right panel). These results are consistent with the previous findings that FGF19 specifically interacts with FGFR4 (21), however, they do not necessarily rule out interactions between FGF19 and other receptors under different conditions. To further dissect the individual roles played by βKlotho and heparin on the interactions between FGF19 and FGFR4, we tested additional combination of heparin and βKlotho. In the absence of βKlotho and heparin, no significant interactions were observed between FGF19 and FGFR4 (Fig. 2B left panel). The addition of either soluble βKlotho conditioned media or heparin alone increased the interaction between FGF19 and FGFR4 (Fig. 2B middle two panels). The strongest signal was detected when both βKlotho and heparin were present in the immunoprecipitation solution (Fig. 2B right panel). These results suggest that although either βKlotho or heparin alone could potentiate interactions between FGF19 and FGFR4, they show an even greater effect and perhaps may work synergistically with FGFR4 to provide the high affinity receptor complex for FGF19.

αKlotho May Also Potentiate FGF19 Signaling. The Klotho component requirements for FGF21
and FGF23 co-receptor complexes appear very specific; FGF21 only works through βKlotho and FGF23 only works through αKlotho (16, 17, 20). We wanted to test the specificity of FGF19 toward the Klotho proteins. An experiment similar to that performed for βKlotho (Fig. 2B) was also established for αKlotho. In the absence of αKlotho and heparin, similar to the results seen in Fig. 2B, no significant interaction was observed between FGF19 and FGFR4 (Fig. 3 left panel). The addition of either soluble αKlotho conditioned media or heparin alone increased the interactions between FGF19 and FGFR4 (Fig. 3 middle two panels). Again, similar to what was observed for βKlotho, the strongest signal was detected when both αKlotho and heparin were present in the immunoprecipitation solution (Fig. 3 right panel). These results suggest that, in contrast to FGF21 and FGF23 which are specific for one form of Klotho protein, FGF19 is able to interact with FGFR complexes that are formed with either αKlotho or βKlotho.

To further investigate the role αKlotho plays in FGF19 signaling, we used Western blot to measure ERK1/2 phosphorylation in HEK293 cells transfected with α or βKlotho treated with recombinant FGF19. As shown in Figure 4, unlike FGF21 and FGF23 which only respond to one of the Klotho proteins (16, 17, 20), FGF19 is able to activate ERK1/2 phosphorylation in cells transfected with either β or αKlotho (Fig. 4). Heparin also seemed to stimulate ERK1/2 phosphorylation in the presence of αKlotho as it did in the presence of βKlotho (Fig. 2B and 4). FGF19 was not able to activate ERK1/2 phosphorylation in HEK293 cells without Klotho proteins (Fig. 1 and data not shown). Therefore, it appears that at least in vitro, FGFRs/heparin complexed with either βKlotho or αKlotho may serve as receptor complexes for FGF19.

Both heparin and heparan sulfate are known to stimulate FGF signal transduction by increasing the affinity between the FGFs and FGFRs (23), however, only heparan sulfate is present on cell surfaces. In our pull down assay, we also found that heparin enhanced the binding of FGF19 and FGFR4 independent of α or βKlotho. However, in the ERK1/2 phosphorylation assay using HEK 293 cells, FGF19 couldn’t activate FGF signaling with heparin alone (Fig. 1). One potential explanation is that although either Klotho or heparin can stabilize the FGF19-FGFR complex, Klotho is fundamentally required for the activation of FGFR by FGF19, perhaps by inducing a specific conformation change in the receptor which activates downstream signaling pathways. The other possibility is that the presence of endogenous heparan sulfate on the cell surface masked the effects of exogenously added heparin at the concentrations tested in the absence of Klotho proteins. To assess the possible contribution of endogenous heparan sulfate to the FGF19 signaling and provide evidence that the observed heparin effects are physiologically relevant, we tested the effects of heparitinase treatment on FGF19 induced ERK1/2 phosphorylation in βKlotho transfected HEK293 cells. As shown in Figure 5, heparitinase treatment significantly suppressed the FGF19 signaling in the absence of added heparin as observed by the reduced ERK1/2 phosphorylation levels. This result suggests that endogenous heparan sulfate could contribute to FGF19 signaling and FGFRs/heparan sulfate complexed with Klotho may serve as receptor complexes for FGF19.

Mice deficient in βKlotho share similar phenotypes with mice lacking either FGF15 (murine ortholog of human FGF19) or FGFR4 (9, 24). In the present study, we show that βKlotho enables FGF19 to bind to FGFR4 and activate ERK phosphorylation. Our results strongly suggest that βKlotho acts as a cofactor that converts a canonical FGFR into a specific receptor for FGF19. The expression pattern of βKlotho may therefore restrict the action of FGF19, and thus confer the tissue-specific bioactivity on FGF19. This would be consistent with studies on the other two family members, FGF21 and FGF23 (16, 17, 20). We also discovered that under our in vitro assay conditions, FGF19 has specificity toward αKlotho as well as βKlotho, although no apparent phenotypic overlapping between FGF19 and αKlotho has been reported. Such dual specificity was not found for FGF21 and FGF23 (16, 17, 20). The physiological relevance of
FGF19 and αKlotho interactions will be a subject for future studies.

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**FIGURE LEGENDS**

**Figure 1.** FGF19 activates HEK293 cells transfected with βKlotho in ERK1/2 luciferase reporter assay. HEK 293 cells were transfected with pFA2-Elk1, pFR-Luc and pRL-TK luciferase reporter plasmids and βKlotho expression vector or GFP control. After starving the cells overnight in DMEM media plus 0.2% BSA, various concentrations of FGF19 was added and the luciferase activity was measured after 5 hours. Heparin was added just before FGF19 treatment. Maximal heparin response was obtained at 20μg/ml, further increasing heparin concentrations up to 200μg/ml had no additional effects.

**Figure 2.** FGF19 interaction with FGFRs requires both βKlotho and heparin. A) FGF19 binds to FGFR4. Interactions between FGF19 and Fc-FGF fusion receptors were analyzed by pull-down assay. In the presence of 20 μg/ml heparin and soluble βKlotho, FGF19 binds to FGFR4. Left panel and right panel are on the same gel with the same exposure. B) FGF19 requires either heparin or βKlotho to bind to FGFR4. βKlotho is needed for the binding between FGF19 and FGFR4 in the absence of heparin. Heparin alone can also enhance the interaction between FGF19 and FGFR4.

**Figure 3.** αKlotho enhances the binding of FGF19 to FGFR4. FGFR4 can interact with FGF19 in the pull down assay in the presence of either αKlotho or heparin.

**Figure 4.** FGF19 actives ERK1/2 phosphorylation in HEK293 cells transfected with αKlotho or βKlotho. HEK293 cells were transfected with expression vectors for αKlotho or βKlotho. After starved with serum-free medium overnight, the cells were stimulated with vehicle or 50 nM recombinant FGF19 for 15 min and snap-frozen in liquid nitrogen. Heparin (20μg/ml) was added just before the FGF19 treatment. Cell lysates were processed for western blot with antibodies against phosphorylated ERK1/2 (pERK1/2) or total ERK1/2 (ERK1/2).

**Figure 5.** Effects of heparitinase treatment on FGF19 activation in HEK293 cells. HEK293 cells were transfected with expression vectors for βKlotho. After serum starved overnight, cells were treated with 0.2 U/ml heparitinase for 3 hours before stimulated with 100 nM recombinant FGF19 for 15 min and snap frozen in liquid nitrogen. Cell lysates were processed for western blot with antibodies against phosphorylated ERK1/2 (pERK1/2) or total ERK1/2 (ERK1/2).
Figure 1

**Figure 2A**

| Heparin | + | + | + | + | + | + | + | + | + | + | + |
| βKlotho | - | - | - | - | - | + | + | + | + | + | + |
| FGFR   | 1c | 2c | 3c | 4 | - | 1c | 2c | 3c | 4 | - |
| FGF19  |   |   |   |   |   |   |   |   |   |   |

**Figure 1**

![Graph showing luminescence against [FGF19], M with different conditions: GFP, GFP+Heparin, βKlotho, βKlotho+Heparin.](image-url)

![Blot images for Heparin, βKlotho, and FGF19](image-url)
### Figure 2B

|          | Heparin | βKlotho | FGFR4 | FGF19 |
|----------|---------|---------|-------|-------|
|          | -       | -       | +     | +     |
|          | -       | +       | -     | +     |
|          | -       | -       | -     | +     |
|          |         |         |       |       |

### Figure 3

|          | Heparin | αKlotho | FGFR4 | FGF19 |
|----------|---------|---------|-------|-------|
|          | -       | -       | +     | +     |
|          | -       | +       | -     | +     |
|          | -       | -       | -     | +     |
|          |         |         |       |       |
### Figure 4

|        | βKlotho |       | αKlotho |       |
|--------|---------|-------|---------|-------|
| Heparin| -       | -     | +       | +     |
| FGF19  | -       | +     | -       | +     |
| p-ERK1/2|         |       |         |       |
| ERK1/2 |         |       |         |       |

### Figure 5

|        |         |       |         |       |
|--------|---------|-------|---------|-------|
| Heparitinase | -       | -     | +       | +     |
| FGF19  | -       | +     | -       | +     |
| P-ERK1/2|         |       |         |       |
| ERK1/2 |         |       |         |       |
Co-receptor requirements for fibroblast growth factor-19 signaling
Xinle Wu, Hongfei Ge, Jamila Gupte, Jennifer Weiszmann, Grant Shimamoto, Jennitte Stevens, Nessa Hawkins, Bryan Lemon, Wenyen Shen, Jing Xu, Murielle Veniant-Ellison, Yue-Sheng Li, Richard Lindberg, Jin-Long Chen, Hui Tian and Yang Li

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