Separating mitogenic and metabolic activities of fibroblast growth factor 19 (FGF19)

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Communicated by Roger D. Kornberg, Stanford University School of Medicine, Stanford, CA, June 30, 2010 (received for review March 31, 2010)

FGF19 and FGF21 are distinctive members of the FGF family that function as endocrine hormones. Their potent effects on normalizing glucose, lipid, and energy homeostasis in disease models have made them an interesting focus of research for combating the growing epidemics of diabetes and obesity. Despite overlapping functions, FGF19 and FGF21 have many discrete effects, the most important being that FGF19 has both metabolic and proliferative effects, whereas FGF21 has only metabolic effects. Here we identify the structural determinants dictating differential receptor interactions that explain and distinguish these two physiological functions. We also have generated FGF19 variants that have lost the ability to induce hepatocyte proliferation but that still are effective in lowering plasma glucose levels and improving insulin sensitivity in mice. Our results add valuable insight into the structure–function relationship of FGF19/FGF21 and identify the structural basis underpinning the distinct proliferative feature of FGF19 compared with FGF21. In addition, these studies provide a road map for engineering FGF19 as a potential therapeutic candidate for treating diabetes and obesity.

FGF21 | FGFR4 | diabetes | hepatocellular carcinoma | hepatocyte proliferation

The FGF19 subfamily members, consisting of FGF19, FGF21, and FGF23, are distinctive members of the FGFs. Most of the FGF family members function in paracrine fashion to regulate processes of development, transformation, and angiogenesis. In contrast, the weak affinity toward heparan sulfate of the pericellular space as well as the presence of intramolecular disulfide bonds allows the FGF19 subfamily members to escape from the extracellular compartment into circulation and to function as endocrine hormones (1–3).

The subfamily members FGF19 and FGF21 share the ability to regulate glucose, lipid, and energy homeostasis (4–7). Both FGF19- and FGF21-transgenic mice are resistant to diet-induced obesity and have decreased body fat mass and improved insulin sensitivity, glucose disposal, and plasma lipid parameters (7, 8). Injection of recombinant FGF19 or FGF21 protein into diabetic mice resulted in the reduction of serum glucose and insulin levels, improved glucose tolerance, and reduced hepatosteatosis and body weight (4–6, 9–11). These effects regarding correction of metabolic imbalances were potent, beneficial, and suggested that FGF19 and FGF21 might be exciting candidates for combating the growing epidemics of diabetes and obesity. Potential unwanted mitogenic activity, however, has been observed in FGF19-transgenic mice, which developed hepatocellular carcinoma within 12 mo and showed increased hepatocyte proliferation as early as 2–4 mo of age (12). The increased hepatocyte proliferation also has been observed in normal mice injected with recombinant FGF19 for 6 d (12); however, such a proliferative effect has not been observed in FGF21-treated animals (13). Therefore, the key distinction between FGF19 and FGF21 is that FGF19 has both metabolic and proliferative effects, whereas FGF21 has only metabolic effects.

The interactions between paracrine-acting FGFs and their receptors have been studied extensively (14, 15). These FGF molecules bind tightly to cell-associated heparan sulfate glycosaminoglycans and exert their actions by forming heparan-mediated high-affinity interactions with FGF receptors (FGFR) and activating receptor tyrosine kinases (16). The FGFRs are encoded by four genes (*FGFR1–FGFR4*), and alternative splicing of *FGFR1–3* generates tissue-specific "b" and "c" isoforms (14, 15).

In contrast to other FGF proteins, FGF19 subfamily members have a weak affinity toward heparan sulfate of the pericellular space, allowing subfamily members to function as endocrine hormones (1, 2). To compensate for the loss of heparan sulfate-mediated high-affinity interactions observed with the receptors of other FGFs, the FGF19 subfamily members instead use single-transmembrane–containing Klotho proteins to facilitate their interactions with and activations of FGFRs (17). There are two related Klotho proteins: αKlotho, which is used as the coreceptor by FGF23, and βKlotho, which is used by FGF19 and FGF21 (17–19). Both Klotho proteins interact only with the "c" isoforms of FGFRs 1–3 and FGFR4, restricting the potential receptor complexes that could be used by this subfamily as well as restricting the potential target tissues to the sites where appropriate Klotho proteins and FGFRs are expressed (19).

Both FGF19 and FGF21 can activate FGFRs 1c, 2c, and 3c in a βKlotho-dependent manner (19, 10). FGF19, however, can activate FGFR4 in the presence of either heparin (used instead of heparan sulfate for in vitro studies) or βKlotho (10). In contrast, FGFR4 activation has not been reported with FGF21 treatment under any condition (10, 19). The ability to activate liver FGFR4 by FGF19 has been proposed to be the signal for enterohepatic regulation of bile acid metabolism (20). We have shown recently that the activation of liver FGFR4 may be responsible for the enhanced hepatocyte proliferation observed with FGF19 treatment (13). However, the structural determinants that differentiate receptor utilization by FGF19 and FGF21 have not been elucidated, nor have the mechanisms that differentiate the metabolic and proliferative effects of FGF19 from those of FGF21.

In the present study, we aim to understand the structural features that determine the distinct functions of FGF19 and FGF21 and in particular the structural features responsible for the proliferative activities of FGF19. We also attempt to determine whether the mitogenic and metabolic activities of FGF19 can be separated to retain the beneficial metabolic effects of FGF19 while reducing its adverse effects on tumorigenicity.

Results

β1-β2 Loop and β10–β12 Region Responsible for Heparin Interaction in Paracrine-Acting FGFs Are also Required for Heparin-Induced FGF19/FGFR4 Interaction/Activation. To understand the basis for differen-

Author contributions: X.W., R.L., Z.W., and Y.L. designed research; X.W., H.G., B.L., S.V., H.B., J.W., J. Gupte, and J. Gardner performed research; X.W., H.G., B.L., S.V., H.B., J.W., J. Gupte, J. Gardner, and Y.L. analyzed data; and X.W. and Y.L. wrote the paper.

Conflict of interest statement: All authors are employees of Amgen, Inc.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1009427107/-/DCSupplemental.

tiating the metabolic effects from the proliferative effects, we first needed to understand the structural features that determine the interaction between FGF19/21 and receptors/cofactors. We have shown previously that a key difference between FGF19 and FGF21 is the ability of FGF19, but not of FGF21, to activate FGFR4. Furthermore, we have shown that the activation of liver FGFR4 is responsible for FGF19-induced hepatocyte proliferation (10, 13) and that FGF19 can activate FGFR4 either through heparin or βKlotho (10). Because FGF21 can activate its receptors only in the presence of βKlotho, it was important to determine whether FGF19-induced hepatocyte proliferation is specific to heparin, to βKlotho-induced FGFR4 activation, or both. Therefore, we first attempted to understand which regions of FGF19 mediate heparin-induced FGFR4 activity.

As revealed by the apo-FGF19 and FGF23 structures [Protein Data Bank (PDB) codes: 2P23 and 2P39, respectively] (1), the β1-β2 loop and β10–β12 regions (the sequences for FGF19 and FGF21 are shown in Fig. 1*A*), which have been shown to be responsible for high-affinity heparin binding in other FGF family members, are very different in this subfamily (1). In the FGF19 and FGF23 structures, the β1-β2 loops and β10–β12 segments are larger and form helical structures. Both these changes result in steric hindrance with heparan sulfate binding and therefore may contribute to the observed weakened interaction of FGF19 subfamily members with heparan sulfate (1). However, it is unclear whether the β1-β2 loop and the β10–β12 regions of FGF19 still may contribute to its weak interactions with heparin and to the heparin-induced FGFR4 activation.

An FGF21 model based on the published apo-FGF19 structure was built and revealed that, in addition to the potential steric clash, the surface charges on FGF21 in these regions also are less favorable for heparin binding (Fig. S1*A*). The FGF21 model also may explain why FGF21 has an even lower affinity than FGF19 for heparin (1) and the lack of any heparin-induced FGF21/FGFR interactions. We explored this difference between FGF19 and FGF21 to study the contribution of the β1-β2 loop and β10–β12 regions to heparin binding by FGF19. Chimeric proteins were constructed in which the heparin-interacting β1-β2 loop and β10–β12 segments in FGF19 were replaced with the corresponding sequences from FGF21 (Fig. 1*B*; the replaced sequences are underlined in Fig. 1*A*). As shown in the solid-phase binding assay, replacing these two regions of FGF19, individually or in combination, abolished heparin-induced FGF19/FGFR4 interaction (Fig. 1*C* and Fig. S1*B*), suggesting that, similar to paracrine-acting FGFs, these two regions in FGF19 may mediate heparin-induced FGF19/FGFR4 interaction.

To validate these findings in the functional assay further, we transfected receptors into L6 cells, which have low levels of endogenous FGFR expression and normally do not respond to FGF treatment. In L6 cells transfected with FGFR1c and βKlotho, both wild-type FGF19 and the chimeric FGF19 molecules (FGF19-1, -2, and -3) are able to activate receptor signaling, as monitored by the ERK phosphorylation levels (Fig. 1*D*, *Left*). This result suggests that all the chimeric molecules in the putative heparin-binding domains are folded properly and are active. Consistent with the solid-phase binding results and in contrast to wild-type FGF19, the substitutions in these putative heparin-binding domains abolished heparin-dependent FGFR4 activation (Fig. 1*D*, *Right*, FGFR4

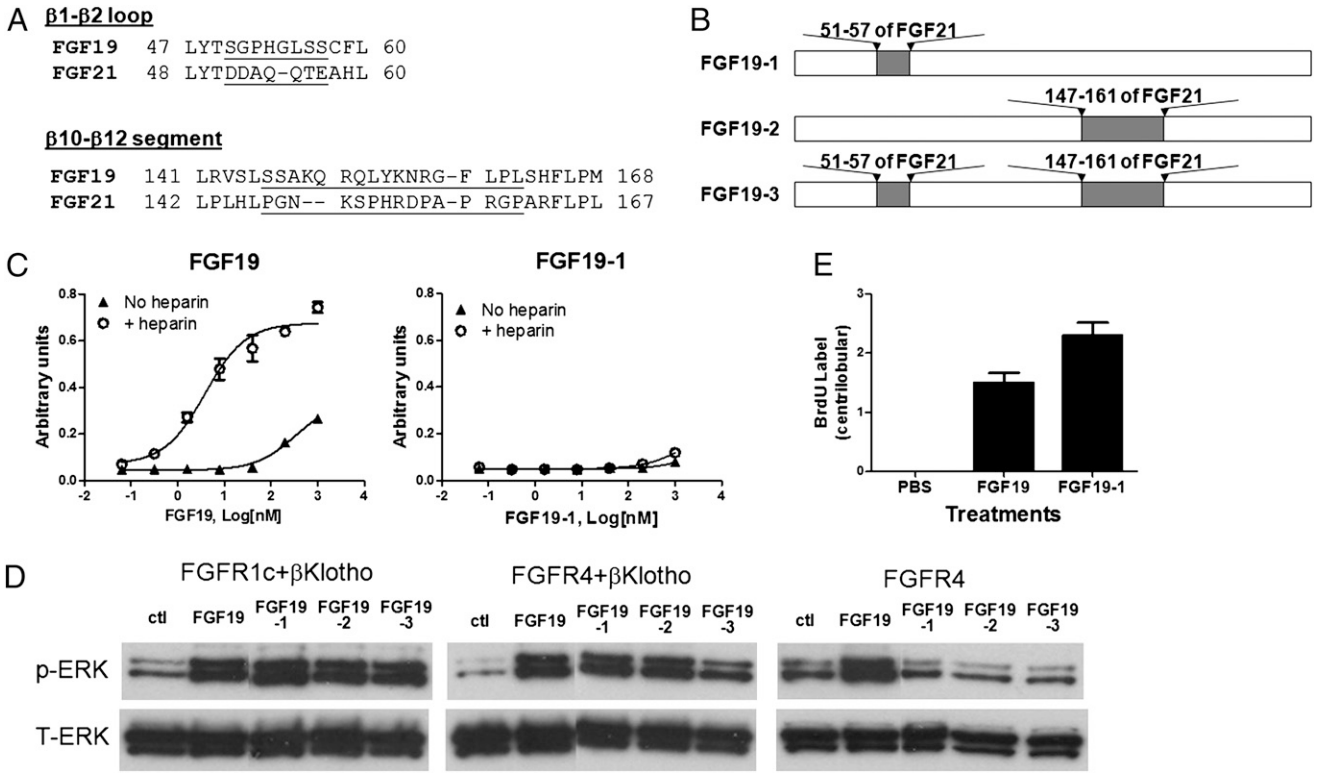

Fig. 1. Effects of mutations in heparin-binding regions of FGF19 on FGFR4 interaction/activation. (*A*) Sequence alignment between FGF19 and FGF21 around the β1-β2 loop and β10–β12 segment. Sequences swapped in the chimeric proteins are underlined. (*B*) Schematic diagram showing chimeric proteins FGF19-1, -2, and -3. (*C*) Solid-phase binding assay measuring the interaction between FGFR4 and FGF19 or FGF19-1 in the presence and absence of heparin. (*D*) L6 cells were transfected with expression vectors for FGFR1c plus βKlotho, FGFR4 plus βKlotho, or FGFR4 alone. After overnight serum starvation, cells were stimulated with vehicle (ctl) or 50 nM recombinant FGF19 or FGF19-1, -2, or -3 for 15 min and were snap-frozen in liquid nitrogen. Cell lysates were prepared for Western blot analysis using antibodies against phosphorylated ERK1/2 (p-ERK) or total ERK1/2 (T-ERK). (*E*) Semiquantitative analysis of BrdU immunostaining of livers from female FVB mice treated for 6 d with PBS, or 2 mg/kg/d recombinant FGF19 or FGF19-1. The scores assigned to BrdU incorporation for these animals were based on a semiquantitative scale described in *Materials and Methods*.

alone transfection), whereas βKlotho-dependent FGFR4 activation was preserved (Fig. 1*D*, *Center*). These results suggest that the mutations in the putative heparin-binding domain indeed selectively abolished heparin-dependent receptor activities. In addition, we have generated FGF19 variants that depend entirely on βKlotho for FGFR4 activation.

To test the effects of βKlotho-dependent FGFR4 activation on hepatocyte proliferation, FGF19-1 was injected into mice, and histopathologic examination of liver sections from FGF19-1–treated animals showed enhanced BrdU labeling in pericentral hepatocytes similar to that seen with FGF19 treatment (Fig. 1*E*). This result suggests that βKlotho-induced FGFR4 activation may cause enhanced hepatocyte proliferation and that removing heparin-induced FGFR4 activity is not sufficient to abolish this undesired mitogenic effect of FGF19. Similar to wild-type FGF19, FGF19-1 still is active in other metabolic assays and is able to induce glucose uptake into adipocyte cells and to reduce plasma glucose levels in an *ob*/*ob* diabetic animal model (Fig. S1 *C* and *D*); this activity suggests that the heparin-domain mutations did not affect FGF19-mediated metabolic regulation.

Residues 38–42 from FGF19 Are Necessary but Not Sufficient to Account for the Ability of FGF19 to Activate FGFR4 and Increase Hepatocyte Proliferation. At the N-terminal region of FGF19, we previously identified a five-amino acid region (residues 38–42) that has been shown to be important for FGFR4 activation (13). The alignment of FGF19 and FGF21 around these five amino acid residues is shown in Fig. 2*A*. The region of FGF21 that corresponds to the five FGF19 residues (WGDPI), contains only three amino acids (GQV). We previously showed that introduction of these five amino acids into the corresponding region of FGF21 (FGF21/$19^{38–42}$; Fig. 2 *A* and *B*) provided gain-of-function activity on FGF21 to activate FGFR4 and to induce increased hepatocyte proliferation (13). To determine whether mutations in these five amino acids are sufficient to abolish the mitogenic effect of FGF19, we next replaced these five residues, ^{38}WGDPI^{42}, from FGF19 with the corresponding sequences, ^{41}GQV^{43}, from FGF21 to generate FGF19/$21^{41–43}$ (Fig. 2*B*). If this region is a necessary component for FGFR4 activation, the substitution of the FGF21 sequence into FGF19 should abolish that activity. Similar to FGF19 and FGF21, FGF19/$21^{41–43}$ induced ERK phosphorylation in L6 cells transfected with FGFR1c and βKlotho (Fig. 2*C*) and was active in adipocyte glucose uptake assays. However, this substitution affected FGFR4 activity only partially, and FGF19/$21^{41–43}$ still retained the ability to induce ERK phosphorylation in L6 cells transfected with FGFR4 and βKlotho (Fig. 2*C*). Furthermore, histopathologic examination of liver sections from FGF19/$21^{41–43}$-treated animals still showed enhanced BrdU labeling in pericentral hepatocytes, similar to that seen in sections from FGF19-treated animals and consistent with our hypothesis that activation of FGFR4 results in mitogenic activity (Fig. S2). The results suggest that removing the N-terminal region alone is not sufficient to abolish the undesired mitogenic effect of FGF19.

One unexpected finding is that FGF19/$21^{41–43}$ is no longer able to activate FGFR4 in the absence of βKlotho (Fig. 2C, *Right*; FGFR4 alone transfection). This observation suggests that heparin-induced FGF19/$21^{41–43}$/FGFR4 activation also has been affected by this substitution. This notion is further confirmed by a solid-phase binding assay in which addition of heparin no longer stimulates FGF19/$21^{41–43}$ interaction with FGFR4 (Fig. 2*D*), similar to the effects observed with mutations in the heparin-binding sites of β1-β2 and β10–β12 regions shown in Fig. 1.

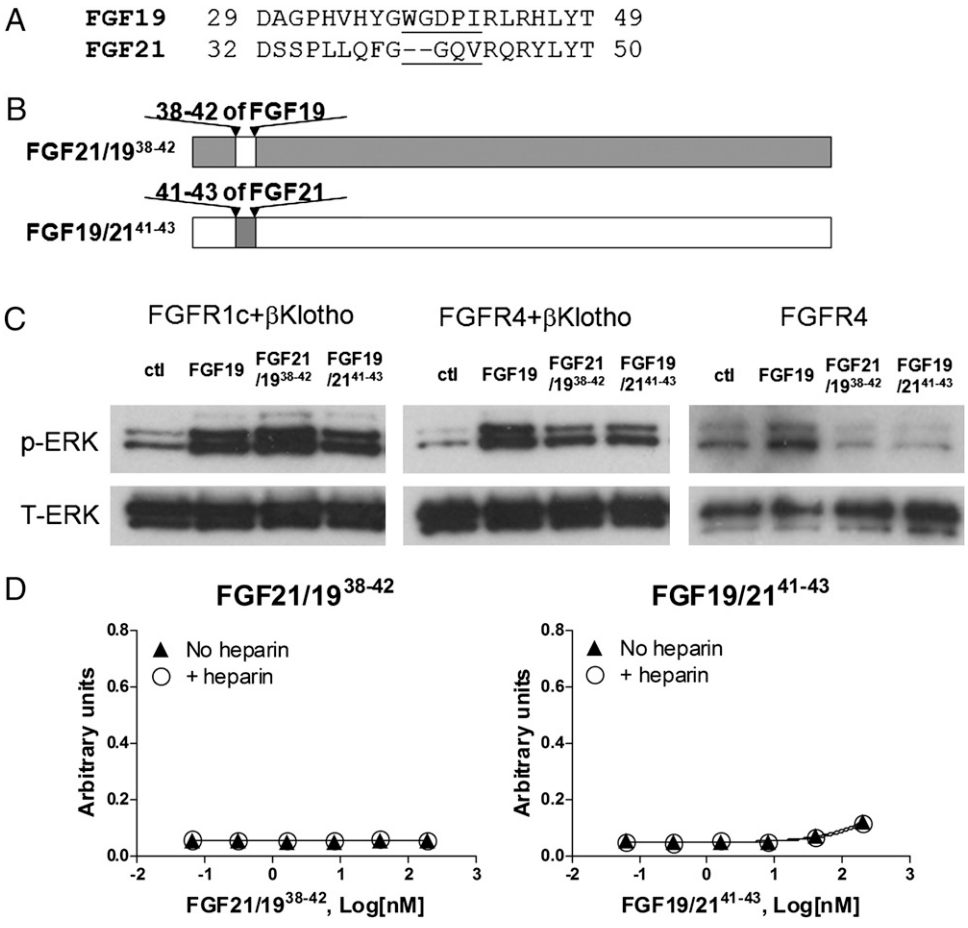

Fig. 2. Effects of residues 38–42 of FGF19 on FGFR4 interaction/activation. (*A*) Sequence alignment between FGF19 and FGF21 near the N-terminal region. Sequences swapped in the chimeric proteins are underlined. (*B*) Schematic diagram showing chimeric proteins FGF21/$19^{38–42}$ and FGF19/$21^{41–43}$. (*C*) L6 cells were transfected with expression vectors for FGFR1c plus βKlotho, FGFR4 plus βKlotho, or FGFR4 alone. After overnight serum starvation, cells were stimulated with vehicle (ctl) or 50 nM recombinant FGF19, FGF21/$19^{38–42}$, or FGF19/$21^{41–43}$ for 15 min and were snap-frozen in liquid nitrogen. Cell lysates were prepared for Western blot analysis using antibodies against phosphorylated ERK1/2 (p-ERK) or total ERK1/2 (T-ERK). (*D*) Solid-phase binding assay measuring the interaction between FGFR4 and FGF21/$19^{38–42}$ or FGF19/$21^{41–43}$ in the presence or absence of heparin.

Combination of Replacing Residues 38–42 from FGF19 and the Heparin-Binding Regions in FGF19 Is Sufficient to Abolish FGFR4 Activation and Hepatocyte Proliferation. Because the N-terminal 38–42 residue region and heparin-binding domains seem to make independent contributions to FGFR4 activation, we next tested the effects on this activity of combining changes in both regions. Therefore, additional chimeric proteins combining FGF19 N-terminal residues 38–42 and one or both of the heparin-interaction regions were constructed (Fig. 3*A*), and their activities were tested in vitro and in vivo. The combined changes in these two distinct regions had minimal impact on the ability of the mutant proteins to activate the FGFR1c/βKlotho receptor complex (Fig. 3*B*, *Left*). However, these combined changes were able to abolish completely the ability of the mutants to activate FGFR4 signaling in L6 cells in either the absence or presence of βKlotho (Fig. 3*B*). Consistent with our proposed hypothesis, histopathologic examination of liver sections from animals treated with the chimeric proteins FGF19-4, -5, and -6 did not show increased BrdU labeling in hepatocytes in pericentral regions, nor was increased BrdU labeling noted in any other area of the liver (Fig. 3*C*). Therefore, the enhanced hepatocyte proliferation associated with wild-type FGF19 had been abolished by the combined mutagenesis of the five N-terminal amino acid residues (38–42) and heparin-binding domains.

Chimeric Molecules That Had Lost the Ability to Activate FGFR4 Still Are Able to Regulate Glucose Homeostasis. Because the chimeric FGF19 molecules FGF19-4, FGF19-5, and FGF19-6 that harbor the combined substitutions in the five N-terminal amino acids (38–42) and one or both of the heparin-binding domains still were able to activate FGFR1c/βKlotho receptor signaling in L6 cells (Fig. 3*B*), we next tested their ability to regulate glucose homeostasis. Their effects on glucose uptake into adipocytes were tested first. Similar to wild-type FGF19 protein, these chimeric proteins demonstrated in vitro ability to stimulate glucose uptake into 3T3L1 adipocytes independent of insulin (Fig. 3*D*). To investigate further the ability of the chimeric proteins to regulate glucose homeostasis, *ob*/*ob* mice were injected i.p. with FGF19 or FGF19-4. Blood glucose levels were measured at 0, 1, 3, and 5 h after injection, and the values are reported as the area under the curve (AUC) means ± SEM over this time period (Fig. 3*E*). Plasma glucose levels were significantly reduced in mice injected with either FGF19 or FGF19-4, with comparable potency and efficacy (Fig. 3*E*). These results suggest that FGF19-4 selectively lost its ability to induce FGFR4-mediated hepatocyte proliferation but retained its ability to modulate glucose regulation.

Discussion

The FGF19 subfamily comprises distinctive FGF family members that function as endocrine hormones to regulate important

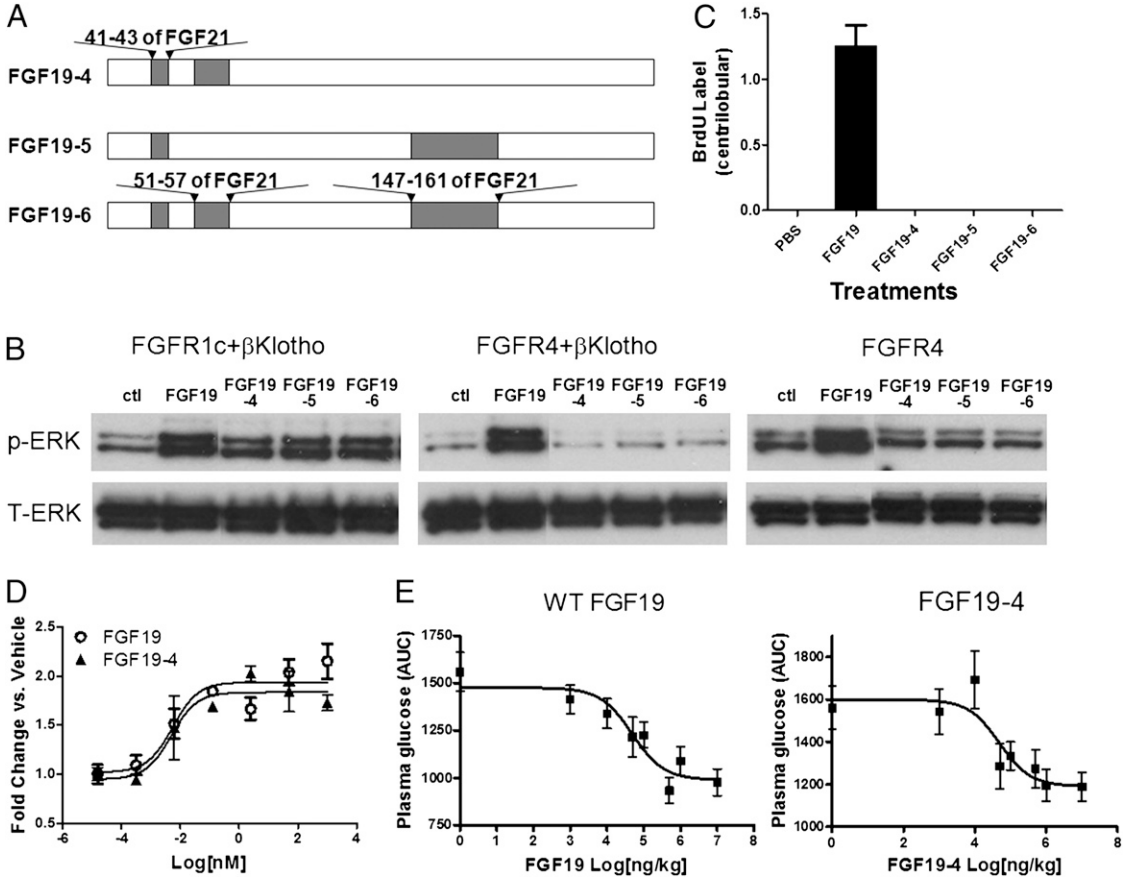

Fig. 3. Combination mutations of residues 38–42 and heparin-binding regions of FGF19. (*A*) Schematic diagram showing chimeric proteins FGF19-4, -5, and -6. (*B*) L6 cells were transfected with expression vectors for FGFR1c plus βKlotho, FGFR4 plus βKlotho, or FGFR4 alone. After overnight serum starvation, cells were stimulated with vehicle (ctl) or 50 nM recombinant FGF19 or FGF19-4, -5, or -6 for 15 min and were snap-frozen in liquid nitrogen. Cell lysates were prepared for Western blot analysis using antibodies against phosphorylated ERK1/2 (p-ERK) or total ERK1/2 (T-ERK). (*C*) Semiquantitative analysis of BrdU immunostaining of livers from female FVB mice treated for 6 d with PBS or with 2 mg/kg/d recombinant FGF19 or FGF19-4, -5, or -6. The scores assigned to BrdU incorporation for these animals were based on a semiquantitative scale described in *Materials and Methods*. (*D*) Differentiated 3T3-L1 adipocytes were incubated for 72 h with recombinant FGF19 or FGF19-4 and assayed for glucose uptake. (*E*) *Ob*/*ob* mice were injected with recombinant FGF19 (n = 10) or FGF19-4 (n = 10). Plasma glucose levels were measured between 3 and 7 h after injection and plotted as AUC during this time period.

physiological functions (4–7, 21). The subfamily members FGF19 and FGF21 have similar abilities to regulate glucose and lipid metabolism but differ significantly in their ability to induce cellular proliferation. FGF19 has been shown to cause hepatocyte proliferation, whereas such an effect has not been observed for FGF21 (12, 13). Therefore, to enable therapeutic development based on these molecules, it is important to understand the mechanistic basis that differentiates these different physiological effects.

We first tried to understand the structural basis for the difference in the ability of FGF19 and FGF21 to activate FGFR4 by cofactor heparin. The surface encompassing the β1-β2 loop and the β10–β12 segment responsible for heparin interaction and heparin-induced FGFR activation in paracrine-acting FGFs varies significantly in the FGF19 structure (16). Our results show that this surface still is important for the weak FGF19/heparin interaction and heparin-induced FGFR4 activation by FGF19. Together with the observation that mutation in these regions reduced the affinity of FGF19 for heparin (1), our results suggest that in FGF19 these regions interact directly with heparin (similar to paracrine-acting FGFs) and also that these regions are responsible for the difference between FGF19 and FGF21 with regard to heparin effects.

The dependence of FGF19 variants harboring mutations in the heparin-binding surface (HBS) on βKlotho for FGFR4 activation allowed us to assess whether βKlotho-induced FGFR activation is distinct from heparin-induced signaling. In vivo, such mutations in the putative heparin domain did not affect the ability of FGF19 to regulate plasma glucose levels, nor did they qualitatively alter the ability of FGF19 to increase hepatocyte proliferation (Fig. 1 and Fig. S1). Therefore, our previous results with a heparin-specific FGFR4 activator, FGF19dCTD (10), and current results with the βKlotho-specific FGFR4 activator, FGF19-1, suggest that with respect to liver FGFR4-induced bile acid regulation and hepatocyte proliferation, both heparin- and βKlotho-mediated FGFR4 activation result in similar qualitative end points.

We previously identified a five-amino acid segment (residues 38–42) in the N-terminal region of FGF19 that contributes to FGFR4 activation (13). The substitution of these five amino acid residues into FGF21 generated FGF21/19$^{38–42}$, which demonstrated a gain-of-function on FGF21 with respect to its ability to activate FGFR4 (Fig. 2 and ref. 13). A model for FGF19/FGFR interaction was built based on the apo-FGF19 structure (PDB code: 2P23) (1) overlaid with the FGF2 complex structure with FGFR1 (PDB code: 1FQ9) (22). Interestingly, this model suggests that the five residues (^{38}WPDPI^{42}) of FGF19 may directly contact the βC′-βE loop of the receptor D3 domain, a region that has been associated with the determination of receptor specificity in paracrine-acting FGFs (Fig. 4 *A* and *C* and ref. 16). An unexpected finding came from FGF19/21$^{41–43}$, which has mutations in the five N-terminal residues (38–42). In contrast to our expectation, the mutations did not abolish βKlotho-induced FGFR4 interaction/activation; however, they did abolish heparin-induced FGFR4 interaction/activation (Fig. 2*C*). Given that the 38–42 region appears to be away from the putative HBS formed by the β1-β2 loop or the β10–β12 segments in the model (Fig. 4*C*), the effect of these five residues on heparin-induced FGFR4 interaction suggests an indirect role, perhaps through an induced shift in position of FGF19 that abolishes HBS interaction with heparin. Future studies will be required to determine whether the 38–42 region interacts directly with heparin and the exact relationship of this region to HBS/heparin interaction. On the other hand, the ability of FGF19/21$^{41–43}$ to activate FGFR4 in a βKlotho-dependent but not in a heparin-dependent manner and the inability of the double mutations in the 38–42 region and HBS surface to activate FGFR4 in the presence of either βKlotho or heparin (Figs. 2*C* and 3*B*) suggest that the HBS also may contribute to βKlotho-induced FGFR4 activation. Interestingly, the modeled structure suggests that the extended HBS might interact directly with the receptor. Together, these results suggest that, similar to paracrine-acting FGFs, the N-terminal region of FGF19 (and, by extension, the N-terminal regions of the other subfamily members, as well) may interact directly with the D3 region of FGFR to achieve receptor specificity and activation. However, distinct contacts between its unique HBS and

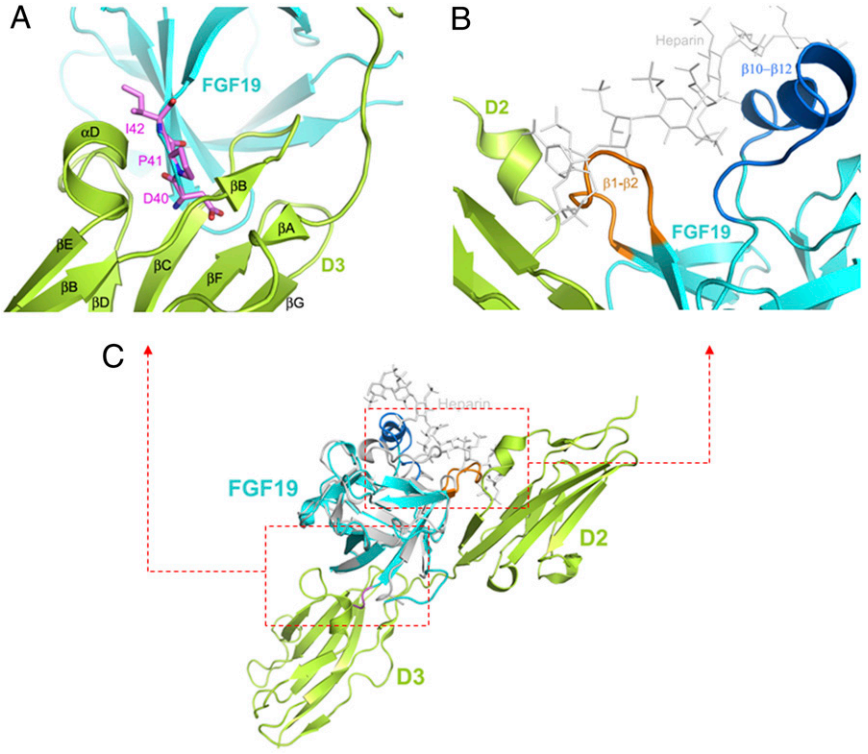

Fig. 4. Structural comparison of FGF19 and FGF2/FGFR1. *(A)* The potential interface between the N-terminal region of FGF19 (magenta) and the Ig-like extracellular domain 3 (D3) of the receptor. (*B*) The potential interface between the β1-β2 (orange) and β10–β12 (blue) segments of FGF19, heparin, and the Ig-like extracellular domain 2 (D2) of the receptor. (*C*) Ribbon representation of FGF19 structure superimposed on the complex structure of FGF2/FGFR1. FGF19 (PDB code: 2P23) is shown in cyan. The N-terminal residues ^{40}DPI^{42} are shown in magenta. In the ternary complex structure (PDB code: 1FQ9), FGF2 is gray, and FGFR1 is green. Heparin is shown by gray sticks.

the receptor D2 region also may contribute to specific receptor interactions.

The observation that the mutations in either of these two opposite regions alone were not sufficient to abolish the overall ability of FGF19 to activate FGFR4 and to induce hepatocyte proliferation suggests that either of these two regions may activate FGFR4 independently and that combined mutations in both regions would be required to abolish this activity completely. Consistent with our hypothesis, the combined mutant FGF19 molecules (FGF19-4, FGF19-5, and FGF19-6; Fig. 3*A*) completely lacked the ability to activate FGFR4 in the presence of either βKlotho or heparin. In contrast, they retained the ability to activate FGFR1c/βklotho (Fig. 3*B*). These mutant FGF19 molecules still were able to induce glucose uptake into adipocytes in vitro and to lower plasma glucose levels in vivo (Fig. 3 *D* and *E*) but had lost the ability to induce liver hepatocyte proliferation, in agreement with our hypothesis that liver FGFR4 activation contributes to hepatocyte proliferation. The impact of these mutant FGF19s on chronic hepatocellular carcinoma formation remains to be evaluated; however, these results are a direct demonstration that the undesirable mitogenic and the beneficial metabolic activities of FGF19 can be separated and that the undesirable mitogenic activity can be removed from FGF19.

By exploring some of the unique properties of FGF19 and FGF21, the studies presented in this report have provided insight into the function of FGF19 subfamily members and allowed us to identify the structural determinants that dictate differential receptor interactions that may explain different physiological functions of FGF19 and FGF21. The ability to switch the five-amino acid N-terminal domain and the two heparin regions without grossly affecting the activity of the new chimeric molecule on the FGFR1c/βKlotho complex, as well as the ability to switch the C-terminal Klotho-interacting domain among subfamily members (23), suggests a modular evolution of these proteins. Nature has evolved many different protein scaffolds to serve diverse biological functions. In some ways, the FGF family could be considered another structural scaffold. As our results suggest, a core sequence forms the scaffold onto which various loops and regions have evolved for specific interactions with FGFRs, coreceptor Klotho, and/or heparin to form an FGF molecule with a particular biological activity. It would be interesting to test whether, by introducing the α- or βKlotho-interacting C-terminal domain and by removing the heparin binding, a paracrine-acting FGF could be converted into an endocrine FGF, and vice versa. This investigation and other interesting FGF protein domain-mapping/engineering studies could be pursued in the future.

These results also may help renew interest in exploring FGF19 as a potential therapeutic candidate for treating diabetes and obesity. FGF21 holds great promise as a treatment for diabetes, but its effectiveness in humans and its potential clinical issues remain unknown; an engineered FGF19 may serve as an ideal complement for FGF21 in diabetes therapy development.

Materials and Methods

Expression and Purification of Recombinant FGF19 and Chimeric Proteins. Wild-type FGF19 (encoding residues 23–216, without the secretory leader peptide sequence) and chimeras were cloned into the pET30 vector (Novagen). DNA constructs were transformed into BL21(DE3) *E. coli* (Novagen). Protein expression was induced with isopropyl β-D-1-thiogalactopyranoside at 37 °C. The purification process was the same as previously described (23). The sequences of all chimeras are as follows:

FGF21/19$^{38\text{-}42}$: M::huFGF21(28-40)::huFGF19(38-42)::huFGF21(44-208); FGF19/21$^{41\text{-}43}$: M::huFGF19(23-37)::huFGF21(41-43)::huFGF19(43-216); FGF19-1: M::huFGF19(23-49)::huFGF21(51-57)::huFGF19(58-216); FGF19-2: M::huFGF19(23-145)::huFGF21(147-161)::huFGF19(163-216); FGF19-3: M::huFGF19(23-49)::huFGF21(51-57)::huFGF19(58-145)::huFGF21(147-161)::huFGF19(163-216); FGF19-4: M::huFGF19(23-37)::huFGF21(41-43)::huFGF19(43-49)::huFGF21(51-57)::huFGF19(58-216); FGF19-5: M::huFGF19(23-37)::huFGF21(41-43)::huFGF19(43-145)::huFGF21(147-161)::huFGF19(163-216); FGF19-6: M::huFGF19(23-37)::huFGF21(41-43)::huFGF19(43-49)::huFGF21(51-57)::huFGF19(58-145)::huFGF21(147-161)::huFGF19(163-216).

The solid-phase binding assay, cell culture and transfections, analysis of FGF signaling, glucose uptake assay, and in vivo hepatocyte BrdU labeling: were carried out as previously described (10, 13).

Mice and Treatment. Age-matched *ob/ob* mice were used in all the studies. Recombinant proteins were injected i.p. into mice at the stated concentration. After the indicated time, blood was collected by tail bleeding, and blood glucose was measure by a glucose meter (Roche Diagnostics). For chronic treatment, mice were injected once daily for 12 d before tissue-collection.

Oral Glucose Tolerance Test. After overnight fasting, mice were injected with a bolus of glucose (1 g/kg body weight) into the stomach by a gavage needle (20G × 1.5 in) (Popper and Sons), and 30 μL of blood was sampled at 0, 15, 30, 60, and 120 min for plasma glucose analyses. Plasma glucose was measured by using a glucose assay reagent (Sigma).

ACKNOWLEDGMENTS. We thank David Penny, Todd Hager, Hossein Salimi-Moosavi, Yi Liu, and Barbara Felder for technical support; Ming Wang, Suzanne Coberly, Jie Tang, Grant Shimamoto, Nigel Walker, Murielle Veniant, Bei Shan, Wen-Chen Yeh, Luke Li, Jackie Sheng, Kevin Moore, and Tom Boone for helpful discussions; and Scott Silbiger and Ming Wang for editing this manuscript.

1. Goetz R, et al. (2007) Molecular insights into the klotho-dependent, endocrine mode of action of fibroblast growth factor 19 subfamily members. *Mol Cell Biol* 27:3417–3428.
2. Asada M, et al. (2008) Glycosaminoglycan affinity of the complete fibroblast growth factor family. *Biochim Biophys Acta* 1780:1432–1440.
3. Wu X, Li Y (2009) Role of FGF19 induced FGFR4 activation in the regulation of glucose homeostasis. *Aging (Albany NY)* 1:1023–1027.
4. Xu J, et al. (2009) Fibroblast growth factor 21 reverses hepatic steatosis, increases energy expenditure, and improves insulin sensitivity in diet-induced obese mice. *Diabetes* 58:250–259.
5. Coskun T, et al. (2008) Fibroblast growth factor 21 corrects obesity in mice. *Endocrinology* 149:6018–6027.
6. Fu L, et al. (2004) Fibroblast growth factor 19 increases metabolic rate and reverses dietary and leptin-deficient diabetes. *Endocrinology* 145:2594–2603.
7. Tomlinson E, et al. (2002) Transgenic mice expressing human fibroblast growth factor-19 display increased metabolic rate and decreased adiposity. *Endocrinology* 143:1741–1747.
8. Inagaki T, et al. (2007) Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21. *Cell Metab* 5:415–425.
9. Xu J, et al. (2009) Acute glucose-lowering and insulin-sensitizing action of FGF21 in insulin resistant mouse models—Association with liver and adipose tissue effects. *Am J Physiol Endocrinol Metab* 297:1105–1114.
10. Wu X, et al. (2009) Selective activation of FGFR4 by an FGF19 variant does not improve glucose metabolism in *ob/ob* mice. *Proc Natl Acad Sci USA* 106:14379–14384.
11. Li X, et al. (2009) Inhibition of lipolysis may contribute to the acute regulation of plasma FFA and glucose by FGF21 in *ob/ob* mice. *FEBS Lett* 583:3230–3234.
12. Nicholes K, et al. (2002) A mouse model of hepatocellular carcinoma: Ectopic expression of fibroblast growth factor 19 in skeletal muscle of transgenic mice. *Am J Pathol* 160:2295–2307.
13. Wu X, et al. (2010) FGF19-induced hepatocyte proliferation is mediated through FGFR4 activation. *J Biol Chem* 285:5165–5170.
14. Ornitz DM, Itoh N (2001) Fibroblast growth factors. *Genome Biol* 2:reviews3005.
15. Zhang X, et al. (2006) Receptor specificity of the fibroblast growth factor family. The complete mammalian FGF family. *J Biol Chem* 281:15694–15700.
16. Mohammadi M, Olsen SK, Ibrahimi OA (2005) Structural basis for fibroblast growth factor receptor activation. *Cytokine Growth Factor Rev* 16:107–137.
17. Kurosu H, et al. (2006) Regulation of fibroblast growth factor-23 signaling by klotho. *J Biol Chem* 281:6120–6123.
18. Wu X, et al. (2007) Co-receptor requirements for fibroblast growth factor-19 signaling. *J Biol Chem* 282:29069–29072.
19. Kurosu H, et al. (2007) Tissue-specific expression of betaKlotho and fibroblast growth factor (FGF) receptor isoforms determines metabolic activity of FGF19 and FGF21. *J Biol Chem* 282:26687–26695.
20. Inagaki T, et al. (2005) Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. *Cell Metab* 2:217–225.
21. Liu S, Quarles LD (2007) How fibroblast growth factor 23 works. *J Am Soc Nephrol* 18:1637–1647.
22. Schlessinger J, et al. (2000) Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Mol Cell* 6:743–750.
23. Wu X, et al. (2008) C-terminal tail of FGF19 determines its specificity toward Klotho co-receptors. *J Biol Chem* 283:33304–33309.