The Autosomal Dominant Hypophosphatemic Rickets R176Q Mutation in Fibroblast Growth Factor 23 Resists Proteolytic Cleavage and Enhances in Vivo Biological Potency*

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Missense mutations in fibroblast growth factor 23 (FGF23) are the cause of autosomal dominant hypophosphatemic rickets (ADHR). The mutations (R176Q, R179W, and R179Q) replace Arg residues within a subtilisin-like proprotein convertase (SPC) cleavage site (RXXR motif), leading to protease resistance of FGF23. The goals of this study were to examine in vivo the biological potency of the R176Q mutant FGF23 form and to characterize alterations in homeostatic mechanisms that give rise to the phenotypic presentation of this disorder. For this, wild type and R176Q mutant FGF23 were overexpressed in the intact animals using a tumor-bearing nude mouse system. At comparable circulating levels, the mutant form was more potent in inducing hypophosphatemia, in decreasing circulating concentrations of 1,25-dihydroxyvitamin D3 (1,25(OH)2D3), and in causing rickets and osteomalacia in these animals compared with wild type FGF23. Parameters of calcium homeostasis were also altered, leading to secondary hyperparathyroidism and parathyroid gland hyperplasia. However, the raised circulating levels of parathyroid hormone were ineffective in normalizing the reduced 1,25(OH)2D3 levels by increasing renal expression of 25(OH)D3-1α-hydroxylase (Cyp27) to promote its synthesis and by decreasing that of 25(OH)D3-24-hydroxylase (Cyp24) to prevent its catabolism. The findings provide direct in vivo evidence that missense mutations from ADHR kindreds are gain-of-function mutations that retain and increase the protein’s biological potency. Moreover, for the first time, they define a potential role for FGF23 in dissociating parathyroid hormone actions on mineral fluxes and on vitamin D metabolism at the level of the kidney.

Renal phosphate wasting is associated with a number of hereditary disorders including X-linked (XLH) and autosomal dominant (ADHR) forms of hypophosphatemic rickets. In addition to hypophosphatemia, patients with these two conditions exhibit decreased or inappropriately normal serum levels of 1,25-dihydroxyvitamin D3 (1,25(OH)2D3), as well as rickets and osteomalacia. Interestingly, ADHR encompasses not only the classic presentation of hypophosphatemia and rickets, but also it displays variable penetrance with delayed onset of the disease and an even more perplexing feature, spontaneous resolution of the biochemical defect (1, 2).

The genes responsible for XLH and ADHR have now been identified. Through positional cloning, a gene that spans the deleted region Xp22.1 in XLH patients or is mutated in nonleision patients with the disorder has been identified (designated PEX and subsequently PHEX, for phosphate-regulating gene with homologies to endopeptidases on the X chromosome) (3). The predicted human PHEX gene product exhibits structural similarity to a family of neutral endopeptidases involved in either activation or degradation of peptide hormones. Therefore, PHEX probably functions as a protease, and it may act by processing factor(s) involved in bone mineral metabolism. Extensive mutation analysis of XLH families has revealed a range of defects in the PHEX gene, all of which appear to be loss of function mutations (4).

The ADHR gene product, on the other hand, is a new secreted member of the fibroblast growth factor family of proteins, FGF23 (5, 6). Missense mutations R176Q, R179W, and R179Q in FGF23 from ADHR kindreds replace Arg residues within a subtilisin-like proprotein convertase cleavage site (5, 6, 8). Whereas native FGF23 protein from culture-conditioned medium resolves as 32- and 12-kDa forms, the mutated proteins are detected only as the 32-kDa band (6, 8), suggesting that these mutations cause the ADHR phenotype possibly by preventing proteolytic cleavage and thereby enhancing the biological activity of circulating FGF23. In turn, FGF23 acts either directly or indirectly to decrease phosphate reabsorption in the proximal nephron, leading to renal phosphate wasting.

Whereas conflicting results have been reported regarding the capacity of wild type and mutant FGF23 forms to alter phosphate transport in vitro (6, 9), there has been indirect evidence to support this action of FGF23. First, FGF23 is abundantly expressed in tumors associated with tumor-induced osteomalacia or TIO, a paraneoplastic disease characteris-
ized by many of the clinical and laboratory characteristics reported in patients with ADHR, such as hypophosphatemia caused by renal phosphate wasting and decreased renal synthesis of 1,25(OH)2D3 (10). Because removal of the responsible tumors normalizes phosphate and vitamin D metabolism, a humoral phosphaturic factor, perhaps FGF23, is thought to be responsible for this syndrome. Second, overexpression of wild type FGF23 in an animal model recapitulates the biochemical and skeletal abnormalities associated with TIO (6). Therefore, it has been postulated that overproduction of wild type FGF23 causes TIO by overcoming subtilisin-like proprotein convertase activity, so that the major fraction of the expressed protein is being secreted as the full-length molecule. In ADHR, on the other hand, mutations in FGF23 prevent proteolytic cleavage, increase its stability, and enhance the biological potency of the circulating protein (6, 8). Experimental proof, however, is lacking.

Here, we undertook to explore the biological actions of FGF23 proteins in a relevant setting (i.e. the intact organism), by overexpressing the R176Q (mFGF23) and wild type forms of FGF23 cDNA in the tumor-bearing nude mouse system. We show that the R176Q substitution is a gain-of-function mutation that is resistant to cleavage and in vivo is able to promote the phenotypic alterations associated with the disorder. In addition, we provide an explanation for the associated peculiarities in vitamin D metabolism by describing, for the first time, a role for FGF23 in dissociating the mineral and vitamin D actions of parathyroid hormone (PTH) at the level of the kidney.

MATERIALS AND METHODS
Cloning Human FGF23—Human FGF23 cDNA was cloned by PCR using oligonucleotide primers derived from the published sequence (GenBank™ accession number AF263537). The forward primer was 5′-CCGACAGGATGCAGGTTT′-3′ (93–112 bp), and the reverse primer encompassed sequences encoding the c-myc epitope (underlined) followed by FGF23 sequences (901–888 bp), 5′-CTACTAGTGTTCAGGTCCCTCTTGAGCACTGTCCAG TTGTTGCTCCCATGGAGGATCAGTGGC-GA-3′. The PCR was conducted using as template aliquots of a cDNA expression library generated from a human mesenchymal tumor (hemangiopericytoma) associated with oncogenous osteomalacia (11). The reaction was heated at 94 °C for 2 min and then cycled at 94 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 min for 35 cycles with a final extension at 72 °C for 10 min. The PCR product (~0.9 kb) was first subcloned into the PGEM-T Easy vector (Promega, WI), restriction digested with EcoRI, and ligated into the EcoRI site of the polylinker region of the mammalian expression vector pcDNA3 (Invitrogen). Its authenticity was confirmed by direct DNA sequencing. The wild type cDNA was then used as template to introduce the point mutation at nucleotide 527 (g→a; R176Q) by site-directed mutagenesis using the Transformer kit (Clontech, Palo Alto, CA). The fidelity of clones was verified by direct sequencing.

Cell Culture—Chinese hamster ovary (CHO-K1) cells were purchased from ATCC (Manassas, VA) and were maintained on Ham’s F-12 medium with 10% fetal calf serum. Cells were stably transfected with pCMV3-myc (1 × 107 cells/0.1 ml of phosphate-buffered saline). The experiment was performed twice.

Serum and Urine Biochemistry—Serum and urine concentrations of calcium, phosphorus, and creatinine and serum alkaline phosphatase activity were determined by routine methods using Sigma Diagnostics reagents (St. Louis, MO). Total urinary maximum reabsorption of phosphate per 0.1 ml of glomerular filtrate was calculated using the nomogram of Walton and Bijvoet (12). Serum-intact PTH and human FGF23 were measured using an enzyme-linked immunosorbsent assay (Immutopics, Inc., San Clemente, CA), whereas 1,25(OH)2D3 determinations were performed using a commercially available radioimmunoassay kit (Immuno- diagnostik Systems).

Ribonuclease Protection Assay (RPA)—cDNA fragment corresponding to nucleotides 1177–1515 of mouse sodium-phosphate cotransporter Npt2 (accession number L33875) (13) was prepared by reverse transcription-PCR from mouse kidney RNA, subcloned, and verified by sequencing. Riboprobes for Npt2 and β-actin (250-bp KpnI-XbaI fragment of the mouse β-actin gene) were prepared by transcription of the subcloned cDNA fragments using T7 RNA polymerase and [α-32P]UTP (800 Ci/mmol; PerkinElmer Life Sciences), and RPA was performed, as per the instructions of the manufacturer (Ambion). Total RNA (10 μg) isolated from kidney with TriPure Isolation Reagent (Roche Molecular Biochemicals), and 20-μg aliquots were fractionated by electrophoresis on a 1% formaldehyde agarose gel, transferred to nylon-cellulose membranes by upward capillary transfer in 20× SSC overnight, and hybridized to the radio labeled cDNA fragment (45% formamide, 10% dextran sulfate, 5× SSC, 1× Denhardt’s solution, and 100 μg/ml salmon sperm DNA) at 42 °C overnight. The membranes were washed in 0.1% SDS plus 2× SSC for 15 min at room temperature with rotation and then in 0.1% SDS plus 0.1× SSC for another 15 min at 60 °C. The autoradiograms were prepared using Kodak BioMax film at ~80 °C with intensifying screens. Quantification of signal intensity on autoradiograms was performed using computerized image software.

Skeletal Radiographs—The distal end of femur was removed and dissected free of soft tissue, and radiographs were taken using a Faxitron model 805 radiographic inspection system (22-kV voltage and 4-min exposure time). Eastman Kodak Co. X-Omat TL film was employed and processed routinely.

Histology—Thyroparathyroidal tissue, kidney, femurs, and tibiae were removed and fixed in PLL fixative (2% paraformaldehyde contain- ing 0.075 M lysine and 0.01 M sodium periodate solution) overnight at 5 °C and processed histologically, as previously described (15). The proximal end of the left tibia was decalcified in EDTA glycerol solution for 5–7 days at 5 °C. Decalcified tibiae and other tissues were dehydrated in a graded series of ethanol after which they were embedded in paraffin on a rotary microtome. The sections were stained with hematoxylin and eosin and immunostained, as described below. Undecalcified, the proximal ends of left tibiae were embedded in LR White acrylic resin (London Resin Company Ltd., London, UK). 1-μm sections were cut on an ultramicrotome. These sections were stained for mineral with the von Kossa and counterstained with Alizarin Red S (0.01% in 0.1 M sodium acetate, pH 5.0) and Gomori’s alizarin red S. Immunohistochemistry—Paraffin sections were stained immunohistochemically for PTH, Npt2, and Cyp40 using the avidin-biotin-peroxidase complex technique. Briefly, primary antibody was applied to tissues overnight at room temperature. Goat serum against PTH (16), affinity-purified rabbit serum against Npt2 (courtesy of M. Knepper, University of California, San Diego, La Jolla, CA), and affinity-purified rabbit serum against Cyp40 (provided by G. Hendy, McGill University, Canada) were employed. As negative control, preimmune serum or Tris-buffered saline was substituted for the primary antibody. After washing, tissues...
were incubated with secondary antibody (biotinylated rabbit anti-goat IgG, biotinylated goat anti-rabbit IgG). Sections were then washed and incubated with the Vectastain ABC-AP reagent (Vector Laboratories, Ontario, Canada) for 45 min. After washing with distilled water, the sections were counterstained with methyl green and mounted with Kaiser's glycerol jelly.

**Computer-assisted Image Analysis**—Computer-assisted image analysis was performed, as previously described (15). The positive and negative areas of von Kossa staining in trabecular and cortical bone were measured by digital image capture and image analysis using Northern Eclipse version 6.0 (Empix Imaging Inc., Mississauga, Canada) image software. The mineralization percentage of trabecular and cortical bone was calculated using the following formula: mineralized bone (%) = von Kossa positive area/von Kossa positive area + von Kossa negative area of bone × 100%

**Statistical Analysis**—Data from image analysis are presented as mean ± S.E. (n = 6). Statistical comparisons were made using a two-way analysis of variance, with p < 0.05 being considered significant.

**RESULTS**

A 0.9-kb fragment corresponding to the FGF23 transcript was amplified from several pools of a cDNA expression library generated from a hemangiopericytoma associated with TIO (Fig. 1A) (11). The addition of the c-myc epitope at the carboxyl terminus of the protein was employed for its subsequent identification and visualization. The R176Q mutation, initially described in a family with ADHR (5), was also introduced in the

**FGF23 cDNA using site-directed mutagenesis.** When transiently transfected into COS-1, OK, or CHO cells, the wild type and mutant forms of FGF23 (mFGF23) were processed, as previously described (results not shown) (8).

To investigate the in vivo biological properties of mFGF23, CHO cells were stably transfected with pcDNA3 mammalian expression vector alone or vector expressing either the myc-tagged wild type or the myc-tagged R176Q form of the protein. Following G418 selection, neomycin-resistant clones were further processed by limited dilution to identify high expressor cells. Shown in Fig. 1B is Western blot analysis of conditioned media from single isolated clones. Abundant secretion of mature FGF23 (32-kDa form) was apparent in both wild type and mutant form-expressing cells, whereas vector-transfected CHO cells did not express the protein. Whereas the wild type form was primarily processed to an amino-terminal fragment (not detected) and a 12-kDa carboxyl-terminal fragment, the mature mutant protein failed to be cleaved and remained intact in the conditioned medium.

**The biological activity of the two FGF23 forms was then assessed following subcutaneous implantation into nude mice of CHO cell clones expressing either FGF23, mFGF23, or CHO cells transfected with vector alone as control (each at a total of 1 × 10⁷ cells).** Using an enzyme-linked immunosorbent assay that detects epitopes within the carboxyl-terminal portion of
human but not murine FGF23, circulating serum levels of the protein were shown to be equivalent in both groups of mice expressing FGF23, whereas none was detectable in the control animals (Fig. 1C). The mice were followed for 45 days, and urine and blood samples were obtained periodically for analyses. Tumor-bearing animals secreting FGF23 and mFGF23 developed profound hypophosphatemia as early as 10 days following implantation (the first time blood samples were obtained) that progressively became even more profound, although at a slower rate, over the next 35 days (Fig. 2A). This alteration was accompanied by inappropriate excretion of urinary phosphate (Fig. 2B), as indicated by the decrease in tubular phosphate reabsorption (12). In addition, as observed in patients with TIO and ADHR, FGF23-overexpressing animals developed reduced serum levels of 1,25(OH)₂D₃ that were inappropriate for the prevailing degree of hypophosphatemia (Fig. 2C). Interestingly, these biochemical alterations were somewhat more pronounced in the mice implanted with CHO cells expressing mFGF23 than in those secreting the wild type form of the protein. However, both were significantly different from those from animals implanted with CHO cells carrying the empty vector.

Based on these observations, we next examined parameters of calcium homeostasis. In all three groups of mice, serum calcium concentration was maintained within the normal range (Fig. 3A). However, distinct differences in the levels of circulating PTH were evident among the three groups of animals, being markedly increased in mice overexpressing the two forms of FGF23 (Fig. 3B). This alteration was accompanied by concomitant decreases in urinary calcium excretion (Fig. 3C)
and increases in serum (Fig. 3D) and bone (Fig. 3E) alkaline phosphatase activity, changes consistent with secondary hyperparathyroidism. This was further confirmed by histological analysis of thyroparathyroidal tissue from these animals (Fig. 3F). Predictably, parathyroid glands from mice expressing FGF23 forms were enlarged, as determined by immunoreactive staining for PTH, compared with glands from animals transplanted with CHO cells carrying the empty vector.

The abnormalities in phosphate, vitamin D, and calcium homeostasis were studied further by first assessing the expression of enzymes aimed at increasing circulating levels of 1,25(OH)2D3 failed to take place. In fact, contrary to its anticipated up-regulation, immunoreactivity for 25(OH)D3-1α-hydroxylase (Cyp24) in the proximal renal tubule was reduced (Fig. 4B), whereas transcript levels of 25(OH)D3-24-hydroxylase (Cyp24) were increased (Fig. 4C) instead of diminished. Again, these inappropriate renal adaptations in Cyp40 and Cyp24 expression were more apparent in mice implanted with CHO-mFGF23 cells, as compared with the wild type FGF23 form, suggestive of the increased biological potency of the mutant protein.

We next examined the long bones of these animals. Histological sections of the epiphyseal region of tibiae are shown in Fig. 5. In comparison with the growth plate of the control animals, the growth plate of mice implanted with CHO cells expressing wild-type FGF23 was wider, more disorganized, and less well mineralized. Once more, these rachitic changes were more apparent in the animals implanted with CHO cells expressing the mutant form of FGF23. Osteomalacic changes were also noted in the metaphyses of these bones (Fig. 6A). Increased unmineralized osteoid was present in specimens from animals expressing FGF23 and was even more abundant in bones from mFGF23-expressing mice. This alteration was evident in both trabecular as well as cortical bone (Fig. 6, B–D). The reduced skeletal mineralization arising from expression of the FGF23 forms was further confirmed from radiographic studies (Fig. 6E).

**DISCUSSION**

FGF23 is mutated in the hereditary renal phosphate wasting disorder ADHR (5). Missense mutations described in the patients with ADHR have been postulated to stabilize the protein by impairing its cleavage and thereby potentially elevating circulating concentrations of FGF23, leading to phosphate wasting (6, 8). Here we show using an in vivo overexpression system that the R176Q form of FGF23 that is resistant to proteolytic cleavage has an equivalent and probably higher
capacity than wild type FGF23 to recapitulate the biochemical and skeletal abnormalities associated with ADHR. Similar findings have recently been reported by other investigators (17). Although it is rather difficult to make direct comparisons on the in vivo potency of the two FGF23 forms, their nearly equivalent circulating concentrations that we describe in our experimental model would tend to add further credence to our conclusion. Hence, the mutant protein has profound effects on phosphate homeostasis and vitamin D metabolism, leading to the concomitant development of renal phosphate wasting, rickets, and osteomalacia.

The phenotypic similarities between ADHR, TIO, and XLH would suggest that deregulation of the same phosphate-regulating pathway, namely FGF23, is involved in the pathogenesis of all three renal phosphate-wasting disorders. Thus, as indicated by our studies here and by others (6), overexpression of wild type FGF23 in TIO is likely to overwhelm the subtilisin-like proprotein convertase processing of the protein, leading to increased circulating levels of the unprocessed 32-kDa FGF23 form with the ensuing biochemical and skeletal alterations. Similarly, it is reasonable to postulate that circulating FGF23 also serves as one of the humoral factor(s) that underlie the etiology of XLH. In this model, FGF23, secreted by one or more tissues, would serve as substrate processed by PHEX enzymatic activity. Consequently, inactivating mutations in PHEX endopeptidase would lead to impaired processing of FGF23 and persistent biological activity that would adversely affect renal phosphate handling, calcitriol synthesis, and bone metabolism. Therefore, overproduction of FGF23 by tumors (as in TIO), mutations that prevent its cleavage (as in ADHR), or mutations that inactivate PHEX (as in XLH) would all increase the level of active circulating FGF23, leading to renal phosphate wasting, hypophosphatemia, rickets, and osteomalacia.

The molecular and physiological mechanisms by which FGF23 and its naturally occurring mutant forms associated with ADHR cause derangement in renal phosphate handling, however, are currently unknown. One possibility is that FGF23 acts directly on the kidney to alter phosphate transport and renal parameters of vitamin D metabolism. Studies addressing this scenario, however, tend to be conflicting, since both confirmatory and negative findings have been reported (6, 10). The difficulty in clearly demonstrating a direct effect of FGF23 on phosphate transport in vitro suggests that perhaps a more complex mechanistic model exists. For example, FGF23 may act as an intermediary in causing phosphaturia, by altering either the expression of PHEX or the expression of its putative substrate or by mobilizing another factor whose nature at present remains unknown.

A somewhat unexpected finding in our study, not described by others (17), was the marked increase in circulating PTH levels in animals bearing FGF23- and mFGF23-secreting tumors. This change was attributed to the observed decrease in calcitriol synthesis, leading to hypocalcemia. The ensuing appropriate rise in PTH secretion would then be aimed toward maintaining calcium homeostasis by increasing bone turnover (increased alkaline phosphatase activity in bone and serum) and decreasing urinary calcium excretion, consistent with secondary hyperparathyroidism. Additional confirmatory support for this argument is provided by the diffuse parathyroid hyperplasia observed histologically in these two groups of animals. Increased circulating levels of PTH have been reported in some patients with TIO (18, 19) and in Hyg and Gy mice, the murine homologs of XLH (20). Although not described in ADHR, a trend toward higher PTH levels has been observed in patients with this disorder (1). The reason for the discrepancy between this observation and our findings can be explained, in part, by the fact that in our animal model deregulated overexpression would tend to raise the circulating levels of the FGF23 forms more profoundly. Conceivably, in patients with ADHR, potential feedback mechanisms are likely to be set in motion so as to restrain excess production of FGF23. Whereas the nature of such mechanisms remains unclear, their existence has been substantiated by the apparent reversal of the phenotype in a number of patients with ADHR (2).

The question then arises as to whether the concomitant increase in circulating PTH levels contributes, at least in part, to the decrease in Npt2 expression at the level of the renal proximal tubule and the inappropriate phosphaturia associated with this condition. Confirmation of this would have to await the completion of similar experiments that are presently under way using nude mice carrying targeted disruption of the Pth gene. Nevertheless, it is rather remarkable that when FGF23 is overexpressed, high circulating PTH levels do not normalize 1,25(OH)2D3 serum concentration. Normally, the enzymes Cyp40 and Cyp24 are very tightly and reciprocally regulated by 1,25(OH)2D3 and PTH (21). 1,25(OH)2D3 activates its own breakdown by strongly inducing Cyp24 while at the same time down-regulating Cyp40 (22). On the other hand, PTH induces Cyp40 while down-regulating Cyp24 expression (23, 24). Here we show that, following FGF23 overexpression, Cyp40 expression remains decreased while, concurrently,
Cyp24 expression is increased, effects that are diametrically opposed to those one would anticipate from the presence of increased PTH and decreased 1,25(OH)₂D₃ circulating levels. Thus, an apparent dissociation of PTH actions at the level of the kidney occurs, whereby the effects of PTH on reabsorbing urinary calcium and perhaps promoting phosphaturia are preserved, whereas Cyp40 and Cyp24 expression are refractory to its action. FGF23, therefore, appears to be directly or indirectly responsible for the inappropriate alterations in the activity of the renal vitamin D-metabolizing hydroxylases observed both in our mice and likely in patients with ADHR, TIO, and XLH. This may also explain, in part, the appropriately normal levels of 1,25(OH)₂D₃ associated with these three disorders despite the prevailing hypophosphatemia, a finding that is unique to them, since in other renal phosphate wasting states, 1,25(OH)₂D₃ synthesis is up-regulated. For example, 1,25(OH)₂D₃ synthesis is appropriately increased by hypophosphatemia in Npt2-null mice (25) and in patients with hypophosphatemia associated with heterozygote missense mutations in the NPT2 gene (26).

In summary, our findings demonstrate the enhanced in vivo biological potency of the R176Q mutant form of FGF23 associated with ADHR compared with the wild type protein and provide a novel perspective on the molecular intricacies that underlie the pathophysiology of hereditary and acquired disorders of renal phosphate wasting.

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