Inactivating mutations of Phex cause X-linked hypophosphatemia (XLH) by increasing levels of a circulating phosphaturic factor. FGF23 is a candidate for this phosphaturic factor. Elevated serum FGF23 levels correlate with the degree of hypophosphatemia in XLH, suggesting that loss of Phex function in this disorder results in either diminished degradation and/or increased biosynthesis of FGF23. To establish the mechanisms whereby Phex regulates FGF23, we assessed Phex-dependent hydrolysis of recombinant FGF23 in vitro and measured fgf23 message levels in the Hyp mouse homologue of XLH. In COS-7 cells, overexpression of FGF23 resulted in its degradation into N- and C-terminal fragments by an endogenous decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone-sensitive furin-type convertase. Phex-dependent hydrolysis of full-length FGF23 or its N- and C-terminal fragments could not be demonstrated in the presence or absence of decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone in COS-7 cells expressing Phex and FGF23. In a reticulolysate system, apparent cleavage of FGF23 occurred with wild-type Phex, the inactive Phex-3'M mutant, and vector controls, indicating nonspecific metabolism of FGF23 by contaminating enzymes. These findings suggest that FGF23 is not a direct Phex substrate. In contrast, by real-time reverse transcriptase PCR, the levels of fgf23 transcripts were highest in bone, the predominant site of Phex expression. In addition, Hyp mice displayed a bone-restricted increase in fgf23 transcripts in association with inactivating Phex mutations. Increased expression of fgf23 was also observed in Hyp-derived osteoblasts in culture. These findings suggest that Phex, possibly through the actions of unidentified Phex substrates or other downstream effectors, regulates fgf23 expression as part of a potential hormonal axis between bone and kidney that controls systemic phosphate homeostasis and mineralization.

X-linked hypophosphatemia (XLH) is a disorder characterized by defective calcification of cartilage and bone, growth retardation, impaired renal tubular reabsorption of phosphate, aberrant regulation of 1,25(OH)2D3 production, and resistance to phosphorus and vitamin D therapy (1). XLH is caused by inactivating mutations of PHEX (2–5), a member of the M13 family of type II cell surface zinc-dependent proteases that include nephrisin, endothelin-converting enzymes 1 and 2 (6, 7), KELL (8), and DINE/X-converting enzyme (9, 10). The mouse Phex cDNA sequence is highly homologous to that of humans (11, 12), and inactivating mutations of Phex are identified in several mouse homologues of XLH, including Hyp, Gy, and Shal mice (10, 13, 14).

Current data indicate that Phex regulates the production and/or degradation of a systemic phosphaturic hormone, referred to as phosphatonin (15). The presence of phosphatonin in XLH/Hyp was detected by parabiosis experiments in which Hyp mice transferred the phosphaturic phenotype to normal mice (16). Studies in parathyroidectomized Hyp mice eliminated parathyroid hormone as the responsible phosphaturic factor (17). Subsequently, cross-kidney transplant studies confirmed the presence of a circulating factor in Hyp mice and excluded the presence of a primary renal phosphate wasting defect (18). The observation that Phex is most highly expressed in cartilage, bone, and teeth (3, 10, 11, 19–22), where it is present in osteoblasts, osteocytes, and odontoblasts, raises the possibility that locally produced substrates in the skeleton are involved in the pathogenesis of XLH. In addition, osteoblasts derived from Hyp mice produce a factor capable of inhibiting sodium-dependent phosphate transport in renal tubular cells (23), implicating bone as a possible source of phosphatonin.

Genetic studies of autosomal dominant hypophosphatemia (ADHR) have identified FGF23 as a likely candidate for phosphatonin (24). FGF23 is an ~30-kDa (251 amino acids) protein with an N-terminal region containing the FGF homology domain and a novel 71-amino acid C terminus. Several studies have confirmed that full-length FGF23 is a phosphaturic hormone (25–27) and that cleavage of FGF23 at the R179 site (R176Q, R179W, and R179Q) prevents cleavage and inactivation of FGF23 (24). Tumor-induced osteomalacia (TIO), also called oncogenic osteomalacia, is an acquired hypophosphatemic disorder with phenotypic features similar to ADHR and XLH (28). FGF23 is also secreted from TIO tumors (27, 29, 30), and removal of the TIO tumor is associated with reductions in circulating FGF23 concentrations and correction of the hypophosphatemia (28, 31). In addition, FGF23 is elevated in some subjects with XLH (28, 31, 32). Serum phosphate concentrations are negatively correlated with circulating FGF23 levels in patients with XLH, suggesting that elevated FGF23 is causing the hypophosphatemia in this disorder as well (31).
The phenotypic similarities among ADHR, XLH, and TIO form the basis for an unproven model to explain their common pathogenesis (33). This model presumes that PHEx degrades active full-length FGF23 into inactive fragments (33) and that ADHR, XLH, and TIO are caused by increased circulating levels of FGF23, which acts as a phosphaturic hormone to inhibit sodium-dependent phosphate uptake in the renal proximal tubule. In this model, FGF23 is increased in ADHR because of mutations in FGF23 that render it resistant to PHEx-dependent cleavage, in XLH because of inactivating mutations of PHEx that prevent the normal degradation of FGF23, and in TIO, because overproduction of FGF23 by the tumor overwhelms the degradation capacity of PHEx.

Currently, three fundamental aspects of this model have been established, namely that mutations preventing the metabolism of FGF23 causes ADHR (33), FGF23 possesses phosphaturic actions (26, 27), and serum concentrations of FGF23 are elevated in some subjects with TIO and XLH (28, 31, 32). The requirement that FGF23 is a substrate for PHEx, however, has not been tested rigorously. Indeed, the data regarding Phex metabolism of FGF23 are conflicting. One report suggests that recombinant Phex may cleave FGF23 at the RXXR motif or a nearby site (34), but other studies have failed to confirm Phex-dependent cleavage of FGF23 (35). In addition, the RXXR motif is the consensus cleavage site for pro-protein convertases (36), and all cell lines and expression systems tested to date for generating recombinant FGF23 contain enzymes capable of metabolizing FGF23 into its N- and C-terminal fragments (27, 35). No studies have investigated the possibility that FGF23 biosynthesis might be increased in XLH/Hyp.

In the current investigations we have generated recombinant FGF23 and Phex and tested whether Phex metabolizes FGF23. In addition, we have examined whether fggf23 expression is increased in the Hyp mouse homologue of XLH.

EXPERIMENTAL PROCEDURES

Generation of FGF23 and Phex Constructs—We amplified human FGF23 cDNA coding sequence from human heart total RNA (Clontech, Palo Alto, CA) by reverse transcriptase PCR similar to previous reports (30). Briefly, 1 μg of total RNA was reverse transcribed using random primers, and PCR was performed with 5′-CAGGTGTTGGGCCCGCCCG forward and 5′-GTGAGACTTGCGGAAGGGG reverse primers. High fidelity platinum Pfx DNA polymerase (Invitrogen) was used in all PCR reactions. A 760-bp PCR product containing sequence from the 5′-end of FGF23 was subcloned into pcDNA3.1-V5-His (Invitrogen) to create a cassette containing FGF23 in-frame with V5 and histidine epitope tags at its C-terminal end (pMT-Phex-WT-V5-His), and Phex-WT-V5-His was subcloned in pFASTBac1 vector.

Preparation of Phex-expressing Membrane Protein—We confirmed the activity of Phex against oligopeptide substrates using recombinant Phex constructs. Crude S9 membranes containing Phex were solubilized with 1% n-dodecyl-β-d-maltoside, collected after centrifugation at 10,000 × g for 15 min and stored at −70 °C in multiple aliquots. The protein content of each sample was determined by the NanoOrange™ protein quantitation kit (Molecular Probes, Eugene, OR).

Cell Culture and Transfections—We evaluated Phex-dependent hydrolysis of recombinant FGF23 in co-transfection and co-culture models. COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. We transiently transfected pFLAG-FGF23-His, pFLAG-FGF23R179Q-His, pFLAG-C-FGF23-His, pFLAG-N-FGF23-His, pcDNA3.1-Phex-V5-His, or corresponding vector control plasmids in COS-7 cells. Transfections were performed using FuGENE 6 transfection reagent (Roche Applied Science) following the manufacturer’s protocols. In addition, COS-7 cells were co-transfected with FGF23 expression constructs (1 μg) and either pcDNA3.1-Phex-V5-His or pcDNA3.1-V5-His vector (1 μg). A FuGENE 6 to DNA ratio of 3:1 μl:μg was maintained for all experiments. The cells and conditioned media were harvested 48 h post-transfection. For the generation of stable cell lines overexpressing FGF23, COS-7 cells were transfected with pFLAG-FGF23-His and cultured in the presence of 1 μg/ml ganciclovir (Roche) for 10 days. The cells were then cultured in the presence of 1 μg/ml ganciclovir and 0.5 μg/ml puromycin for an additional 10 days. The cells were harvested and plated on 10-cm tissue culture dishes (ATCC, Manassas, VA) containing 5 mm β-glycerophosphate and 25 μg/ml ascorbic acid as reported previously (38).

Assessment of FGF23 Hydrolysis in Culture—To assess endogenous FGF23 hydrolysis in the absence of Phex, we collected total cell lysates and conditioned media from COS-7 cells after transient and stable transfaction with FGF23 expression constructs. To assess Phex-dependent hydrolysis of FGF23, we collected conditioned media from COS-7 cells following co-transfection with pFLAG-FGF23-His and pcDNA3.1-Phex-V5-His constructs. In addition, we plated COS-7 cells producing FGF23 (1 × 10⁶ cells/well) with COS-7 cells expressing Phex (1 × 10⁶ cells/well) or cells transfected with the control vector and incubated for 24 h in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. The co-cultures were washed with Hank’s balanced salt solution, and conditioned media were collected after incubation for 48 h in serum-free Dulbecco’s modified Eagle’s medium. The conditioned medium was centrifuged at 7000 × g for 10 min to remove cells and cellular debris, and FGF23 hydrolysis was monitored by Western blot analysis (see below). For the inhibitor studies, COS-7 cells stably transfected with pFLAG-FGF23-His were seeded in 6-well plates. After 48 h the medium was replaced with serum-free media containing different concentrations of the furin inhibitor dec-RVKR-cmk (39, 40) (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA), or equivalent amounts of Me₂SO vehicle. The cells were cultured for an additional 24 h and then the conditioned medium was collected for Western blot analysis.

FGF23 Cleavage by Rabbit Reticulocyte Lysates—To evaluate Phex-dependent hydrolysis of FGF23 in vitro, we generated recombinant proteins using the rabbit reticulocyte lysate system. For these studies, pcDNA3.1-Phex-V5-His and pcDNA3.1-Phex-3′-M-V5-His, pcDNA3.1-Phex-3′-I-V5-His, and pcDNA3.1-Phex-3′-Apa-I-V5-His were transfected into rabbit reticulocyte lysates using the TNT T7 Quick Coupled Transcription/Translation System (Promega) following the manufacturer’s instructions. Aliquots (5 μl) of the reaction from the in vitro translated pcDNA 3.1/V5-His vector, full-length, or 3′ mutant Phex were mixed 1:1 with human wild-type or R179Q mutant FGF23-FLAG, and the mixture was incubated for 1 h at 37 °C. The incubation mixtures were subsequently analyzed by immunoblot using anti-V5 antibody conjugated to horseradish peroxidase (Invitrogen) and visualized by enhanced chemiluminescence (ECL).

Assessing Phex Enzyme Activity in Vitro—We assessed Phex activity...
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Fig. 1. Expression of recombinant hFGF23 in COS-7 cells. A, schematic of full-length FGF23, R179Q (the cleavage-resistant FGF23 mutant), and the N- and C-terminal FGF23 fragments. All constructs are tagged at the N terminus with FLAG and the C terminus with the His epitopes. B, Western blot analysis detecting full-length and fragments of rFGF23 expressed in COS-7 cells. Constructs in A were transfected as described under “Experimental Procedures.” Only the full-length FGF23 is identified as a 32-kDa product in total cell lysates (lane 1), but conditioned media contain both full-length and the 22-kDa N-terminal fragment as detected by the anti-M2 antibody (lane 2) and the 18-kDa C-terminal fragment as detected by the anti-His antibody (lane 6). The R179Q mutant is not metabolized when secreted into conditioned media (lane 3). Transfection of N- (lane 4) and C-terminal (lane 5) fragments results in their secretion without further hydrolysis. Variation in band intensity between lanes reflects differences in transcription efficiency.

Fig. 2. Evidence for furin-dependent cleavage of hFGF23 in COS-7 cells. A, schematic of full-length FGF23, 5–50 µM dec-RVKR-cmk was added to FGF23-expressing COS-7 cells, and condition medium was collected after 24 h and analyzed by Western blot with anti-M2 antibody as described under “Experimental Procedures.”

For dot blot analysis of Phex expression was detected by using anti-V5 antibody (Invitrogen). Recombinant FGF23 was detected by using either anti-FLAG-M2 antibody (Sigma-Aldrich) or anti-His horseradish peroxidase conjugates (Qiagen, Inc., Valencia, CA). Phex expression was detected by using anti-V5 antibody (Invitrogen).

RNA Isolation—We isolated RNA from various mouse tissues by grinding snap frozen tissues in liquid nitrogen and then extracting total RNA using Tri reagent (Molecular Research Center, Inc., Cincinnati, OH). RNA samples, pretreated with DNase, were further cleaned using an RNeasy spin column (Qiagen, Inc., Valencia CA), and the yield was quantified using a Ribogreen RNA quantitation kit (Molecular Probes, Eugene, OR).

Quantitative real-time PCR—1.5 µg of total RNA was denatured for 5 min at 65 °C in the presence of 0.5 mol of random hexamer, snap cooled in ice water, and then reverse transcribed in 100 µl using the TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. PCR reactions contained 100 ng of template (cDNA or RNA), 300 nM each forward and reverse primer, and 1× SybrGreen PCR Master Mix (Applied Biosystems, Foster City, CA) in 50 µl. Samples were amplified for 40 cycles in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) with an initial melt at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. PCR product accumulation was monitored at multiple points during each cycle by measuring the increase in fluorescence caused by the binding of SybrGreen I to double-stranded DNA. The partial cycle at which a statistically significant increase in fg/f23 product was first detected (threshold cycle; Ct) and normalized to the Ct for cyclophilin. A passive reference dye, ROX, was used to normalize for variations in volume and/or dye concentration between sample wells. Post-amplification melting curves were performed to confirm that a single PCR product was produced in each reaction. The contribution of contaminating genomic DNA to the observed product was determined from the Ct given by the RNA template. This quantity was usually less than 0.1%. The primer sequences used include FGF23 forward primer 5′-ACTGTGTCAGAAGCCATC-3′ and FGF23 reverse primer 5′-TGGGCGACAGTTGTAGAA-3′. The primer sequences used include FGF23 forward primer 5′-ACTGTGTCAGAAGCCATC-3′ and FGF23 reverse primer 5′-TGGGCGACAGTTGTAGAA-3′.

Statistics—We evaluated differences between groups by one-way analysis of variance for multiple comparison or by Student’s t test for comparisons between two groups. All values are expressed as mean ± S.E. The relationship between gene expressions was assessed by regression analysis. All computations were performed using the Statgraphic statistical graphics system (STSC, Inc., Rockville, MD).

RESULTS

FGF23 Is Processed at the RXR Site—To assess FGF23 hydrolysis, full-length, N- and C-terminal fragments and a cleavage-resistant form of FGF23 containing the R179Q muta-
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**Fig. 3.** Failure to detect Phex-dependent cleavage of recombinant FGF23 in mammalian cells. A, Phex-containing expression vector or an empty vector was co-transfected with wild-type FGF23, its cleavage-resistant FGF23 R179Q mutant, or the N- or C-terminal fragments into COS-7 cells. Endogenous enzyme activity in COS-7 cells results in cleavage of FGF23 into two fragments. There is no evidence for Phex-dependent hydrolysis of full-length FGF23 or its fragments. B, effect of Phex on FGF23 cleavage in the presence and absence of 50 μM dec-RVKR-cmk. The furin inhibitor dec-RVKR-cmk inhibits cleavage of FGF23 by endogenous enzymes in COS-7 cells resulting in the secretion of the full-length FGF23 protein into conditioned media. Co-expression of Phex in COS-7 cells with FGF23 does not result in cleavage of the full-length FGF23 product. C, co-culture of COS-7 cells expressing Phex with COS-7 cells secreting FGF23 showing cleavage of FGF23 by endogenous enzymes independent of the presence of Phex in the culture system. Western blot analysis was performed on cell lysates for Phex (upper panels in A and B) using anti-V5 antibodies and conditioned media for FGF23 using anti-M2 antibodies as described under “Experimental Procedures.”

**A Chloromethyl Ketone-based Inhibitor of Furin-type Convertase Blocks FGF23 Cleavage**—To determine whether the observed cleavage of FGF23 in COS-7 cells was mediated by endogenous pro-protein convertases, we evaluated whether the furin inhibitor dec-RVKR-cmk prevented cleavage of FGF23. dec-RVKR-cmk (5–50 μM) added to the culture media resulted in a dose-dependent inhibition of FGF23 processing in COS-7 cells (Fig. 2), indicating that endogenous furin-type convertase are responsible for FGF23 metabolism.

FGF23 Is Not Cleaved by Phex in Mammalian Cells—To examine whether Phex metabolizes FGF23, we co-expressed Phex and FGF23 in COS-7 cells and assessed cleavage by Western blot analysis (Fig. 3). The co-expression of Phex and full-length FGF23 in COS-7 cells did not alter the ability of the endogenous furin-like enzyme to cleave FGF23 into its N- and C-terminal fragments (Fig. 3A). In addition, co-expression of Phex with the cleavage-resistant R179Q FGF23 mutant did not result in any degradation products. To investigate the possibility that FGF23 might require processing before Phex-mediated hydrolysis, we co-expressed Phex with an expression vector containing either the N-terminal or C-terminal FGF23 fragments in COS-7 cells. Phex also failed to hydrolyze either the N- or C-terminal FGF23 fragments, suggesting that the putative Phex cleavage sites identified in FGF23 (43) are not accessible to the recombinant Phex enzyme under the conditions studied. To exclude the possibility that both Phex and an endogenous furin-like enzyme were cleaving FGF23 at the RXR site, we co-transfected COS-7 cells with full-length FGF23 and Phex in the presence and absence of the furin inhibitor dec-RVKR-cmk (Fig. 3B). Consistent with the prior findings (Fig. 1), the addition of dec-RVKR-cmk blocked the degradation of FGF23 in COS-7 cells (Fig. 3B). In the presence of dec-RVKR-cmk, Phex did not result in cleavage of FGF23. Finally, to exclude the possibility that co-expression of Phex and FGF23 in the same cell might somehow limit enzyme-substrate interactions, we co-cultured COS-7 cells expressing Phex with COS-7 cells producing FGF23. Similar to co-expression studies (Fig. 3A), we found no evidence for Phex-dependent cleavage of FGF23 in co-culture experiments (Fig. 3C).

To confirm the activity of the recombinant Phex, we tested the ability of cell membrane fractions expressing Phex to hydrolyze two different oligopeptide substrates reported to be cleaved by Phex (35, 41). We found that membranes expressing rPhex cleaved the hexapeptide VPQSDS, whereas membranes from vector controls did not cleave this peptide (Fig. 4A). In addition, membranes expressing rPhex hydrolyzed the substrate ZAAL-pNA (Fig. 4B). To rule out the possibility that dec-RVKR-cmk inhibition of Phex might account for the negative results of Phex-dependent cleavage of FGF23, we tested the ability of this inhibitor on Phex-dependent cleavage of the synthetic substrate ZAAL-pNA (Fig. 4, B and C). dec-RVKR-cmk did not block Phex-dependent cleavage of ZAAL-pNA (Fig. 4B), making it unlikely that dec-RVKR-cmk prevented Phex-dependent cleavage of FGF23 in the preceding experiments (Fig. 3). In addition, we found that Phex-dependent cleavage of ZAAL-pNA could be blocked by EDTA but not by phosphoramidon (Fig. 4C).

**Nonspecific Cleavage of FGF23 Occurs in Vitro**—To re-examine prior reports that rPhex hydrolyzes FGF23 in vitro (34), we generated recombinant Phex and FGF23 using a reticulocyte system and incubated the resulting proteins in vitro to assess cleavage (Fig. 5). For these studies mouse full-length (Phex-
WT and C-terminal truncated Phex (Phex-3’M), human wild-type or R179Q mutant FGF23, and pcDNA 3.1/V5-His vector were transcribed in vitro and translated in rabbit reticulocyte lysates. We found that incubation with reticulolysates from wild-type Phex, the inactive Phex-3’M mutant, and vector alone resulted in apparent cleavage of FGF23 as evidenced by the reduction in the intensity of the band for both the wild-type (Fig. 5A) and the FGF23 R179Q mutation (Fig. 5B). The failure to observe Phex-specific cleavage products indicates that the reduction in band intensity is because of the presence of contaminating enzymes in the reticulolysate system.

**Fgf23 Expression Is Increased in Hyp Mouse and Correlates with Increased Mepe Expression in Bone**—Because we were unable to confirm Phex-dependent metabolism of FGF23, we explored whether *fgf23* expression might be increased in the *Hyp* mouse homologue of XLH that has a 3’ deletion of Phex. First, we examined *fgf23* expression by real-time PCR in the normal tissues, including bone, where Phex is predominately expressed (Fig. 6). In normal mice, the rank order of *fgf23* expression in normal mouse tissues was bone > thymus > brain > heart > skeletal muscle > spleen > skin > lung > testes, with nearly undetectable levels in liver and kidney (Fig. 6A). Next, we compared *fgf23* expression in different skeletal sites in normal and *Hyp* mice (Fig. 6B). We found that normal mice express *fgf23* in calvaria, mandible, and diaphysis but extremely low levels in bone marrow. *Hyp* mice expressed markedly increased *fgf23* levels in the calvaria, mandible, and diaphysis compared with normal mice. The increase in *fgf23* in *Hyp* mice was limited to bone, because no increases of *fgf23* were observed in bone marrow (Fig. 6B) or kidney, lung, and liver (data not shown) derived from *Hyp* mice.

To compare the increase of *fgf23* with *mepe*, another bone-related gene reported to be increased in *Hyp* mice (44), we measured *fgf23* and *mepe* expression in calvaria from *Hyp* and normal control mice (Fig. 7, A and B). Although the overall abundance of *mepe* transcripts were higher than *fgf23*, the magnitude of the increase in *fgf23* was greater than that observed for *mepe* (Fig. 7). Of potentially greater significance was the very strong correlation between *fgf23* and *mepe* (Fig. 7C), suggesting that *fgf23* might regulate *mepe* expression or that both are regulated by a common upstream factor resulting from Phex deficiency.

**FGF23 Is Expressed in Hyp-derived Osteoblasts Cell during Maturation**—We previously isolated and characterized immortalized osteoblast cell lines derived from normal wild-type and *Hyp* mice, respectively designated TMOb-Nl and TMOb-Hyp (38). Both TMOb-Nl and TMOb-Hyp osteoblasts undergo a temporal sequence of maturation, but TMOb-Hyp, which lack a functional Phex, display an intrinsic mineralization defect in culture. We examined *fgf23* expression by real-time PCR in TMOb-Hyp and TMOb-Nl osteoblasts during a 14-day culture period during which these cells undergo maturation (Fig. 8). We observed low levels of expression of *fgf23* in TMOb-Nl osteoblasts that were not significantly altered during the maturation sequence from 5 through 14 days of culture. In contrast, in TMOb-Hyp osteoblasts we observed progressive culture duration-dependent increase in *fgf23* expression that peaked at day 10 of culture and then declined during the latter stages of differentiation as these cells transitioned to mature osteoblasts.
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DISCUSSION

A single enzyme-substrate hypothesis has been proposed to explain the role of PHEX and FGF23 in the pathogenesis of XLH (33). This model presumes that the intact FGF23 protein is the physiologically relevant substrate for Phex and that decreased degradation of FGF23 by mutated PHEX accounts for an increase in the circulating levels of this phosphaturic hormone in XLH. The current investigations, however, do not support this hypothesis. Instead, we show that FGF23 is cleaved by a furin-like enzyme at the RXRR site (see Figs. 1 and 2) rather than by Phex (Fig. 3). In contrast, we fail to demonstrate Phex-dependent cleavage of intact FGF23 or its N- and C-terminal fragments using recombinant Phex and FGF23 proteins expressed in mammalian cells (Fig. 3) or using proteins synthesized in vitro (Fig. 5). The inability to show Phex-dependent cleavage of FGF23 is not because of lack of activity of the recombinant Phex enzyme, because we show activity against synthetic oligopeptide substrates (Fig. 4), as reported previously (41, 43). Instead of Phex-dependent degradation, we show that inactivating mutations of Phex result in increased expression of FGF23 transcripts in the bone and cultured osteoblasts of the Hyp mouse homologue of XLH (see Figs. 6–8), indicating that Phex may somehow regulate the biosynthesis of FGF23.

Of these findings, the demonstration that the skeleton is the predominant site of $\text{fgf23}$ expression in normal mice and that $\text{fgf23}$ transcripts are markedly increased in the skeleton of Hyp mice (Fig. 6) are potentially the most important. Prior studies reported $\text{fgf23}$ expression in low abundance in several normal tissues similar to our findings (Fig. 6A) but did not examine $\text{fgf23}$ expression in bone or the effect of Phex deficiency on the levels of $\text{fgf23}$ transcripts (27). In Hyp mice lacking a functional Phex, increased levels of $\text{fgf23}$ transcripts were observed in mandible, calvaria, and diaphysis of the long bone (Fig. 6B). The increase in $\text{fgf23}$ message in Hyp mice was limited to the skeletal sites, because levels of $\text{fgf23}$ expression in bone marrow and other tissues of Hyp mice were similar to levels of $\text{fgf23}$ found in normal mice. Although the present studies did not define the cell type(s) in bone tissue that expresses $\text{fgf23}$, it is likely that osteoblasts and/or osteocytes are the source of $\text{fgf23}$ expression in bone, because differential expression of $\text{fgf23}$ between normal and Hyp mice was not observed in bone marrow (Fig. 6B). In addition, recent studies in patients with the McCune-Albright syndrome have identified FGF23 production by fibrous dysplasia osteoprogenitors and normal bone-forming cells in vivo and in vitro (45). Consistent with the predominant expression of $\text{fgf23}$ in osteoblasts, additional results demonstrate $\text{fgf23}$ transcripts in osteoblast cultures (Fig. 8). More importantly, similar to the increased expression of $\text{fgf23}$ in Hyp bone, $\text{fgf23}$ expression was greater in Hyp compared with normal osteoblasts. The increase in $\text{fgf23}$ in Hyp-derived osteoblasts was most evident at day 10 of culture (Fig. 8), at the transition between the maturation of pre-osteoblasts to mature osteoblasts when Phex expression is normally up-regulated.

Fig. 6. Tissue survey of $\text{fgf23}$ mRNA expression in mice. A, $\text{Fgf23}$ message levels in normal mouse tissues. B, comparison of $\text{fgf23}$ expression in normal wild-type (NL) and Hyp-derived skeletal tissues (Hyp). $\text{Fgf23}$ was predominantly expressed in bone and markedly increased in Hyp compared with normal bone at all sites tested. Expression was assessed by real-time PCR using RNA derived from normal wild-type and Hyp mouse tissues as indicated. Values represent single determinations in A and the mean $\pm$ S.E. (minimum of three normals and eight Hyp) in B. $\text{Fgf23}$ expression is relative to the level of the cyclophilin control gene.

Fig. 7. Comparison of $\text{fgf23}$ and mepe expression in normal wild-type and Hyp-derived calvaria. A, $\text{Fgf23}$ mRNA was markedly increased in Hyp compared with normal calvaria. B, Mepe, a more abundant transcript, was also significantly increased in Hyp compared with normal calvaria. Values represent the mean $\pm$ S.E. of three normals and seven Hyp-derived calvaria. The asterisk denotes a significant difference at $p < 0.05$. C, regression between $\text{fgf23}$ and mepe in normal and Hyp calvaria. A significant correlation between $\text{fgf23}$ and mepe levels was observed ($p = 0.002$).
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Fig. 8. Expression of fgf23 mRNA in normal and Hyp-derived osteoblasts. Immortalized osteoblasts from normal wild-type (TMOB-NI) and Hyp (TMOB-Hyp) mice were cultured for up to 14 days in the presence of ascorbic acid and β-glycerophosphate to induce differentiation as described previously (35). Fgf23 expression was assessed by real-time reverse transcriptase PCR as described under “Experimental Procedures.” TMOB-Hyp osteoblasts expressed higher levels of fgf23 at 7, 10, and 12 days of culture. Values represent the mean ± S.E. of three wells per group. Values with different superscript letters are significantly different at p < 0.0001.

Our prior studies failed to detect fgf23 transcripts in the same osteoblastic cell lines (35), possibly because of the use of less sensitive detection methods and examination of osteoblasts at stages of lowest fgf23 expression. Although fgf23 transcripts are detected in osteoblast cultures, the level of expression in culture is lower than in skeletal tissue. The lower expression in cell lines might be explained by the loss of in vivo stimuli required for maintenance of FGF23 expression or by the expression of fgf23 in other cell types in bone. Thus, the skeleton appears to be the major site of FGF23 expression in health and disease.

Nevertheless, the study of age- and sex-matched Hyp mice and their wild-type littermates more clearly establishes an association between fgf23 expression and Phex deficiency than has been observed in cross-sectional evaluations of subject with XLH (28, 31, 32), a setting in which variable circulating levels has been observed in cross-sectional evaluations of subject with XLH (28, 31, 32), have amino acid sequences that would be predicted to be cleaved by Phex. Indeed, quenched fluorescence peptides derived from FGF23, as well as peptides derived from MEPE, are hydrolyzed by rPhex in vitro (43). In the current study, the failure to demonstrate that full-length FGF23 containing putative cleavage sites are hydrolyzed by Phex, and a prior study (46) that also failed to show Phex-dependent hydrolysis of full-length MEPE, suggest that the mere presence of consensus cleavage sites for an enzyme in a protein does not necessarily indicate that these site are available to the enzyme. Indeed, the inability of Phex to hydrolyze intact FGF23 or MEPE may indicate a constraint placed by incompatible three-dimensional structures that limit enzyme substrate interactions (48, 49). Consequently, large proteins such as FGF23 and MEPE might require processing into smaller fragments before being cleaved by Phex, or other proteins such as Dentin matrix protein 1 may have conformations necessary for Phex-dependent hydrolysis (49). The latter possibility seems more likely, because neither N- nor C-terminal FGF23 fragments are hydrolyzed by Phex (see Figs. 1 and 3). Moreover, the processing of FGF23 leads to its inactivation (26), and to date, biological activities have not been ascribed to the N- and C-terminal FGF23 fragments. Thus, even if Phex could be shown to hydrolyze the N- and C-terminal FGF23 fragments under different conditions, the biological significance of further degradation of these inactive fragments is uncertain.

Finally, the in vitro finding that a furin-like enzyme present in COS-7 cell membranes cleaves FGF23 into N- and C-terminal fragments (see Figs. 1 and 2) is of uncertain physiological significance. Similar degradation by cell surface convertases in vivo might be expected to limit the biological activity of FGF23. It is possible that other factors may be present in the circulation that bind to and prevent FGF23 cleavage that are not present in the in vitro culture systems. Although only full-length FGF23 has been shown to have phosphaturic actions (50), additional studies are needed that investigate its in vivo metabolism and pharmacokinetics.

In conclusion, the increased expression of fgf23 in the skeleton of Hyp mice suggests that increased synthesis may be an important mechanism of elevated circulating FGF23 levels in XLH. In addition, the failure to confirm fgf23 hydrolysis by Phex leaves open the possibility that physiologically relevant Phex substrates remain to be discovered. Taken together, these data support a more complex model, with intermediate steps linking PHEX to FGF23, to explain the pathogenesis of XLH. One possibility is that increased fgf23 expression in the Hyp skeleton results from the actions of yet to be identified Phex substrate or from other downstream consequences of inactivating mutations of Phex. Furthermore, additional direct or indirect effects of fgf23 and Phex to modify mepe expression and metabolism may also explain the intrinsic defect in osteoblast-mediated mineralization that is independent of hypophosphatemia. Whatever the exact mechanism, the interactions between Phex and fgf23 likely occur within the skeleton, where both are expressed. This implicates bone as an endocrine organ
that produces a hormone, fgf23, to regulate renal phosphate conservation to meet the need for bone mineralization.

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REFERENCES

1. Rasmussen, H., and Anast, C. (1983) Familial Hypophosphatemic Rickets and Vitamin D-dependent Rickets, pp. 1743–1773, McGraw-Hill Book Co., New York
2. The HYP Consortium (1995) Nat. Genet. 11, 130–136
3. Guo, R., and Quarles, L. D. (1995) J. Bone Miner. Res. 10, 1009–1017
4. Holm, I. A., Huang, X., and Kunkel, L. M. (1997) J. Bone Miner. Res. 12, 790–797
5. Holm, I. A., Huang, X., and Kunkel, L. M. (1997) J. Biol. Chem. 272, 15262–15268
6. Lee, S., Russo, D. C., Reiner, A. P., Lee, J. H., Sy, M. Y., Telen, M. J., Judd, W. J., Simon, P., Rodrigues, M. J., Chabert, T., Poole, J., Jovanovic-Lorenz-Depiereux, B., Grabowski, M., Meitinger, T., and Strom, T. M. (2002) J. Clin. Endocrinol. Metab. 87, 4957–4960
7. Meraldi, P., Rice, D. C., Richmond, B. A., Ciszewski, A. L., Desbarats, M., Glorieux, F. H., and Ecarot, B. (2001) J. Bone Miner. Res. 16, 13729–13737
8. Takeuchi, Y., Fujita, T., Fukumoto, and S., Yamashita, T. (2002) J. Bone Miner. Res. 17, 1102–1110
9. Takeuchi, Y., Fujita, T., Fukumoto, and S., Yamashita, T. (2002) J. Bone Miner. Res. 17, 1102–1110
10. Beck, L., Soumounou, Y., Martel, J., Krishnamurthy, G., Gauthier, C., Pollack, S., and Soffer, D. (2001) J. Bone Miner. Res. 16, 13729–13737
11. Econs, M. J., and Drezner, M. K. (1994) Biochem. Biophys. Res. Commun. 201, 635–639
12. Holm, I. A., Huang, X., and Kunkel, L. M. (1997) J. Bone Miner. Res. 12, 790–797
13. Holm, I. A., Huang, X., and Kunkel, L. M. (1997) J. Bone Miner. Res. 12, 790–797
14. Meyer, R. A., Jr., Henley, C. M., Meyer, M. H., Morgan, P. L., McDonald, A. G., and Glorieux, H. F. (1997) Am. J. Pathol. 150, 1685–1691
15. Meyer, R. A., Jr., Henley, C. M., Meyer, M. H., Morgan, P. L., McDonald, A. G., Mills, C., and Price, D. K. (1998) Genomics 48, 289–295
16. Ntayi, C., Lorimier, S., Berthier-Vergnes, O., Horneback, W., and Bernard, P. (2003) J. Clin. Invest. 99, 1209–1216
17. Oefner, C., D’Arey, A., Hennig, M., Winkel, F. K., and Dale, E. (2000) J. Bone Miner. Res. 21, 334–349
18. Qin, C., Brunn, J. C., Cook, R. G., Orkiszewski, R. S., Malone, J. P., Veis, A., and Butler, W. T. (2003) J. Bone Miner. Res. 17, 708–719
19. Qin, C., Brunn, J. C., Cook, R. G., Orkiszewski, R. S., Malone, J. P., Veis, A., and Butler, W. T. (2003) J. Bone Miner. Res. 17, 708–719
20. Rouchon, A. F., Marcinkiewicz, M., Siegfried, G., Tenenhouse, H. S., DesGroseillers, S., Levene, C., Yahalom, V., and Redman, C. M. (2001) J. Biol. Chem. 276, 27281–27289
21. Ruchon, A. F., Marcinkiewicz, M., Siegfried, G., Tenenhouse, H. S., DesGroseillers, S., Levene, C., Yahalom, V., and Redman, C. M. (2001) J. Biol. Chem. 276, 27281–27289
22. Smith, M. M., Shi, L., and Navre, M. (1995) J. Bone Miner. Res. 10, 1453–1459
23. White, K. E., Evans, W. E, O’Riordan, J. L. H., Speer, M. C., Econs, M. J., Lorenz-Degireux, B., Grabowski, M., Mettinger, T., and Strom, T. M. (2000) Nat. Genet. 26, 345–349
24. Bai, X. Y., Miao, D., Goltzman, D., and Karaplis, A. C. (2003) J. Biol. Chem. 278, 9843–9849
25. Sato, H., Kasano, K., Kinosaki, M., Ito, H., Hirata, M., Sugawara, H., Miyamoto, K. I., and Fukushina, N. (2000) J. Biol. Chem. 275, 22006–2211
26. Shimada, T., Mizutani, S., Muto, T., Yoneya, T., Hino, R., Takeda, S., Takeuchi, Y., Fujita, T., Fukumoto, S., and Yamashita, T. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6500–6505
27. Yoneya, T., Oka, K., Takeuchi, Y., Fujita, T., Fukumoto, S., and Yamashita, T. (2002) Endocrinology 143, 3179–3182
28. Yoneya, T., Oka, K., Takeuchi, Y., Fujita, T., Fukumoto, S., and Yamashita, T. (2002) Endocrinology 143, 3179–3182