Fibroblast Growth Factor (FGF)-23 Inhibits Renal Phosphate Reabsorption by Activation of the Mitogen-activated Protein Kinase Pathway

The homeostasis of the plasma phosphate level is essential for many biological processes including skeletal mineralization. The reabsorption of phosphate in the kidney is a major determinant of the plasma levels of phosphate. Phosphatonin is a hormone-like factor that specifically inhibits phosphate uptake in renal proximal epithelial cells. Recent studies on tumor-induced osteomalacia suggested that phosphatonin was potentially identical to fibroblast growth factor (FGF)-23. However, as purified recombinant FGF-23 could not inhibit phosphate uptake in renal proximal epithelial cells, the mechanism of action of FGF-23 remains to be elucidated. Therefore, we examined the mechanism of action of FGF-23 in cultured renal proximal epithelial cells, opossum kidney cells. FGF-23 was found to require heparin-like molecules for its inhibitory activity on phosphate uptake. FGF-23 binds to the FGF receptor 3c, which is mainly expressed in opossum kidney cells, with high affinity. An inhibitor for tyrosine kinases of the FGF receptor, SU 5402, blocked the activity of FGF-23. FGF-23 activated the mitogen-activated protein kinase (MAPK) pathway, which is the major intracellular signaling pathway of FGF. Inhibitors of the MAPK pathway, PD98059 and SB203580, also blocked the activity of FGF-23. The present findings have revealed a novel MAPK-dependent mechanism of the regulation of phosphate uptake by FGF signaling.

Phosphate is a nutrient essential for many biological processes including skeletal mineralization and energy metabolism (1). The homeostasis of the plasma phosphate level is essential for these processes. The reabsorption of phosphate in the kidney is a major determinant of the plasma phosphate level. Reabsorption is largely regulated by the type II sodium-dependent phosphate (Na/P) cotransporter that is expressed in renal proximal epithelial cells (1). The activity of the type-II Na/P cotransporter is regulated by hormones, such as parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D (1.25(OH)2D), which have opposite effects. PTH and 1,25(OH)2D decrease and increase the reabsorption of phosphate in renal proximal tubules, respectively (1).

Tumor-induced osteomalacia is a renal phosphate-wasting disorder resulting in low serum phosphorus concentration and osteomalacia. Removal of the tumors responsible for tumor-induced osteomalacia normalizes phosphate metabolism. The responsible tumors secrete a heat-sensitive molecule of ~25 kDa designated as “phosphatonin” that specifically inhibits sodium-dependent phosphate transport in cultured renal proximal epithelial cells. Recent studies on tumor-induced osteomalacia revealed that phosphatonin was potentially identical to fibroblast growth factor (FGF)-23, which is a new member of the FGF family (2, 3, 5). Autosomal dominant hypophosphataemic rickets is also a renal phosphate-wasting disorder resulting in low serum phosphorus concentration, rickets, and osteomalacia. The ADHR gene was also potentially identified to be FGF-23 with missense mutations (4). However, as purified recombinant FGF-23 could not inhibit phosphate uptake in renal proximal epithelial cells (3), the mechanism of action of FGF-23 on the phosphate uptake in renal proximal epithelial cells remains to be elucidated. Therefore, we examined the mechanism of action of FGF-23 on phosphate transport in the cultured renal proximal epithelial cell line, opossum kidney (OK) cells, using purified recombinant FGF-23. The results reported here revealed a novel mechanism of phosphate reabsorption regulated by activation of the mitogen-activated protein kinase pathway mediated by FGF signaling.

EXPERIMENTAL PROCEDURES
Preparation of Purified Mouse FGF-23—The mouse FGF-23 cDNA with a DNA fragment (75 bp) encoding an E tag (GAPVYPDPLEPR) and a hexamer His tag (HHHHHHH) at the 3′ terminus of the coding region was constructed in a transfer vector, pBacPAK 9 (CLONTECH). Recombinant baculovirus containing the FGF-23 cDNA with the tag sequences was obtained by cotransfection of Sf9 cells with recombinant pBacPAK 9 and a Bsu361-digested expression vector, BacPAK6 (CLONTECH), as described (5). High Five insect cells were transfected with the resultant recombinant baculovirus and infected at 27 °C for 72 h in TC-100 insect medium (Invitrogen) containing 10% fetal calf serum. Recombinant mouse FGF-23 was purified from the culture medium by affinity chromatography using Ni-NTA-agarose (Qiagen) and desalted with gel filtration chromatography using Bio-Gel P6 DG (Bio-Rad Lab) in phosphate-buffered saline containing 100 μg/ml bovine serum albumin.

Effects of FGF-23 on Phosphate Uptake in Opossum Kidney Cells—OK cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium/F12 (Invitrogen) with 10% fetal calf serum and seeded in 6- or 96-well dishes. The phosphate uptake in OK cells was determined essentially according to the method described previously (6). OK cells in monolayers were cultured at 37 °C for 24 h in Dulbecco’s modified Eagle’s medium/F12 in 24-well dishes. OK cells were further cultured at 37 °C for 3 h in Dulbecco’s modified Eagle’s medium/F12 containing 1–10 ng/ml FGF-23 and 10 μg/ml heparin. In the experiments using inhibitors specific for the protein kinases of FGF receptor, SU 5402...
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(TCbiochem), and for the mitogen-activated protein kinases, PD98059 and SB203580 (Calbiochem), the inhibitors (25 μM SU 5402, 50 μM PD98059, and 10 μM SB203580) were added to the culture medium for 2 h before treatment with FGF-23. The cultured cells were quickly washed with uptake solution containing 137 mM NaCl, 5.4 mM KCl, 1 mM CaCl2, 1.2 mM MESO4, 15 mM HEPES (pH 7.4) once. Phosphate uptake was initiated by addition of uptake solution supplemented with 0.1 mM KH2PO4/K2HPO4 (1 μCi/ml). The cells were cultured at 37 °C for 5 min. Phosphate uptake was terminated by quickly removing the uptake solution and washing three times with ice-cold stop solution containing 137 mM NaCl and 14 mM HEPES (pH 7.4). The cells were solubilized with 1% Triton X-100, and the cell lysates were used for radioactivity determination with a liquid scintillation counter.

Quantitative Analysis of Opossum Type-II Na/Pi Cotransporter mRNA Expression in OK Cells by Real-time Quantitative Polymerase Chain Reaction—RNA was extracted from OK cells in 6-well dishes using an RNA extraction kit (Qiagen). OK cell cDNA was synthesized in a reaction mixture (20 μl) containing Moloney murine leukemia virus reverse transcriptase, a random hexadeoxynucleotide primer, and OK cell RNA as the template. The type-II Na/Pi cotransporter cDNA was amplified from the cDNA by real-time quantitative polymerase chain reaction (PCR) using a Model 7700 Sequence Detector (PE Applied Biosystems) with a forward primer, reverse primer, and a TaqMan probe specific for opossum type-II Na/Pi cotransporter cDNA (8). Opossum GAPDH cDNA as a control was also amplified with primers for opossum GAPDH cDNA. The copy numbers of the type-II Na/Pi cotransporter and GAPDH cDNAs were determined according to the manufacturer’s instructions.

Isolation of Opossum FGFR-3b and -3c cDNAs—Opossum FGFR-3 cDNA encoding immunoglobulin-like domain III with the surrounding regions was amplified from OK cell cDNA by PCR with primers for mouse FGF-23 cDNA (9). The amplified cDNA was cloned into the pGEM-T DNA vector (Promega). The nucleotide sequences of the clones were determined.

Determination of FGFR-3b and -3c mRNAs Expressed in OK Cells—Opossum FGFR-3 cDNA encoding immunoglobulin-like domain III with the surrounding regions was amplified from OK cell cDNA by PCR with specific primers as described above. The amplified DNA was digested with MspI followed by 8% polyacrylamide gel electrophoresis. Thereafter, the gel was stained with ethidium bromide.

Preparation of Purified Extracellular Domain of Mouse FGFR-3c—The cDNA encoding the extracellular domain of mouse FGFR-3c with a DNA fragment (75 bp) encoding an E tag and a hexameric His tag at the 3’ terminus of the coding region was cloned into the transfer vector, pBacPAK6. Recombinant baculovirus containing the FGFR-3c cDNA with the tag sequence was obtained by cotransfection of Sf9 cells with recombinant pBacPAK6 and a Bsu36I-digested expression vector BacPAK6 as described (7). High Five insect cells were transfected with the resultant recombinant baculovirus and infected at 27 °C for 72 h in EX-Cell 400 complete medium. The recombinant extracellular domain of FGFR-3c was purified from the culture medium by affinity chromatography with Ni-NTA-agarose and desalted by gel filtration chromatography with Bio-Gel P6 DG in phosphate-buffered saline containing 100 μg/ml bovine serum albumin.

Detection of Tyrosine Phosphorylation of Extracellular Signal-regulated Kinase (ERK) and p38 Mitogen-activated Protein Kinase (MAPK)—OK cells in 6-well dishes were lysed with TNE buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 50 mM NaF, 2 mM NaVO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/ml pepstatin. Cell lysates were separated by 8% SDS-polyacrylamide gel electrophoresis under reducing conditions and transferred onto Hybond-ECL nitrocellulose membranes (Amersham Biosciences). Phosphorylated ERK or p38-MAPK was detected by Western blotting analysis using rabbit antibodies against phospho-ERK or phospho-p38 MAPK (New England Biolabs).

RESULTS

Preparation of Purified Mouse Recombinant FGF-23—The mouse FGF-23 cDNA with a DNA fragment encoding an E tag and a hexameric His tag at the 3’ terminus was constructed in the transfer vector. High Five insect cells infected with the recombinant baculovirus efficiently secreted recombinant FGF-23 as described previously (5). Recombinant FGF-23 was purified from the culture medium by affinity chromatography with Ni-NTA-agarose. Recombinant FGF-23 was analyzed by SDS-polyacrylamide gel electrophoresis followed by both protein staining and Western blotting analysis with anti-E tag antibodies. Recombinant FGF-23 showed a major protein band of ~30 kDa (data not shown). The observed molecular mass was essentially consistent with the calculated molecular mass of recombinant FGF-23 (28,034 Da).

Effects of FGF-23 on Phosphate Uptake in Renal Proximal Epithelial Cells—Purified FGF-23 was shown not to inhibit sodium-dependent phosphate uptake in the cultured renal proximal epithelial cell line, OK cells (3). We also examined the ability of FGF-23 to inhibit sodium-dependent phosphate uptake in OK cells by incubation for 3 h with FGF-23. FGF-23 (10 ng/ml) alone could not inhibit phosphate uptake (Fig. 1), consistent with the observations reported previously (3). As heparan sulfate or heparin was reported to modulate the activity of several FGFs (12, 16), we examined the inhibitory activity of FGF-23 in the presence of heparin. In the presence of heparin (10 μg/ml), FGF-23 significantly inhibited phosphate uptake (Fig. 1). We also examined the inhibitory activity of FGF-2 in the presence of heparin. However, FGF-2 could not inhibit phosphate uptake (data not shown).

FGF-23 rapidly (within ~1 h) inhibited phosphate uptake, and the extent of inhibition gradually increased thereafter (Fig. 2). As the phosphate uptake was mainly mediated by the type II sodium-dependent phosphate (NaPi2) cotransporter (8), we examined the effects of FGF-23 on the levels of the type II NaPi2 cotransporter mRNA in OK cells by real-time quantitative PCR. FGF-23 inhibited phosphate uptake without significant changes in the type II NaPi2 cotransporter mRNA levels within 3 h (Fig. 2). These results indicated that FGF-23 inhibits phosphate uptake by regulation of the type-II Na/Pi cotransporter activity at the posttranscriptional level, at least in the short term (~3 h).

Expression of FGFR Receptor-3 in OK Cells—FGFRs exert their activity by binding to FGFRs. There are four types of FGFR gene, FGFR-1, -2, -3, and -4 (12). Renal proximal epithelial cells

Fig. 1. Effects of FGF-23 on phosphate uptake in OK cells. OK cells were treated with different concentrations of FGF-23 for 3 h in the presence or absence of heparin. After treatment, phosphate uptake in OK cells was examined for 5 min.
were shown to preferentially express FGFR-3 (10). Therefore, we examined the expression of FGFR-3 in OK cells. By alternative splicing of the immunoglobulin-like domain III of FGFR-3, two isoforms of FGFR-3, FGFR-3b and FGFR-3c, can be generated (9). As the opossum FGFR3 gene has not been identified, we attempted to amplify opossum FGFR-3 cDNA including the alternative splicing region of the immunoglobulin-like domain III by PCR using OK cell cDNA as a template and primers corresponding to the sequences conserved in mouse and human FGFR-3 cDNA (9). DNA of the expected size (470 bp) was amplified. The amplified DNA was cloned into the pGEM-T vector, and the nucleotide sequences of more than 20 clones were determined. Two different amino acid sequences were predicted from their nucleotide sequences. The sequences of major and minor clones were highly similar to those of mouse FGFR-3c and -3b (9), respectively (Fig. 3), indicating that FGFR-3c was expressed as the major isoform of FGFR-3 in OK cells. To confirm this, the amplified DNA was digested with MspI followed by polyacrylamide gel electrophoresis. Digestion of the amplified FGFR-3b and -3c cDNAs with MspI should yield two DNA fragments of 385 and 90 bp and three DNA fragments of 276, 103, and 90 bp, respectively. The results of MspI-digestion also indicated that FGFR-3c was the major isoform of FGFR-3 expressed in OK cells (Fig. 4).

Binding of FGF-23 to FGFR-3c—To examine whether FGF-23 could bind to FGFR-3c, the recombinant extracellular domain of mouse FGFR-3c was prepared. We examined the binding of FGF-23 to the extracellular domains of FGFR-1c and FGFR-2c. FGF-23 was found to bind to FGFR-2c but not to FGFR-1c (data not shown).
Effects of an Inhibitor Specific for Protein Kinases of FGFR on Inhibition of Phosphate Uptake Induced by FGF-23—SU 5402 is an inhibitor specific for protein kinases of FGFR but not for those of other receptors, platelet-derived growth factor receptor, insulin receptor, and epidermal growth factor receptor (11). SU 5402 is a specific competitor for the ATP-binding site of FGFR. As FGF-23 was found to bind to FGFR-3c with high affinity, we examined whether the inhibition of phosphate uptake by FGF-23 was mediated through the protein kinase activity of FGFR. SU 5402 completely blocked the inhibitory activity of FGF-23, indicating the involvement of activation of the protein kinases of FGFR in inhibition of phosphate uptake by FGF-23 (Fig. 6).

Effects of FGF-23 on Phosphorylation of Extracellular Signal-regulated Kinase and p38 Mitogen-activated Protein Kinase—The major intracellular signaling pathway of FGF was shown to be the Ras/MAPK pathway (12). Therefore, we examined the effects of FGF-23 on phosphorylation of MAPKs, ERK, and p38 MAPK in OK cells by Western blotting analysis using antibodies against phospho-ERK and phospho-p38 MAPK. PD98059 (PD) and SB203580 (SB) are inhibitors of the upstream kinases of ERK and p38 MAPK, respectively. PD (50 μM) and SB203580 (10 μM) were added to the culture medium for 2 h before FGF-23 or FGF-2 treatment. After treatment, phospho-ERK and p38 MAPK were detected by Western blotting analysis.

Effects of an Inhibitor Specific for Protein Kinases of FGFR on Inhibition of Phosphate Uptake Induced by FGF-23—SU 5402 is an inhibitor specific for protein kinases of FGFR but not for those of other receptors, platelet-derived growth factor receptor, insulin receptor, and epidermal growth factor receptor (11). SU 5402 is a specific competitor for the ATP-binding site of FGFR. As FGF-23 was found to bind to FGFR-3c with high affinity, we examined whether the inhibition of phosphate uptake by FGF-23 was mediated through the protein kinase activity of FGFR. SU 5402 completely blocked the inhibitory activity of FGF-23, indicating the involvement of activation of the protein kinases of FGFR in inhibition of phosphate uptake by FGF-23 (Fig. 6).

Effects of FGF-23 on Phosphorylation of Extracellular Signal-regulated Kinase and p38 Mitogen-activated Protein Kinase—The major intracellular signaling pathway of FGF was shown to be the Ras/MAPK pathway (12). Therefore, we examined the effects of FGF-23 on phosphorylation of MAPKs, ERK, and p38 MAPK in OK cells by Western blotting analysis using antibodies against phospho-ERK and phospho-p38 MAPK. FGF-23 was found to significantly induce phosphorylation of both ERK and p38 MAPK (Fig. 7). We also examined the effects of FGF-2 on phosphorylation of MAPKs in OK cells. In contrast to FGF-23, FGF-2 was found to significantly induce phosphorylation of ERK but not that of p38 MAPK (Fig. 7).
Effects of MAPK Inhibitors on Inhibition of Phosphate Uptake by FGF-23—To examine the functional relevance of the activation of MAPKs, we examined the effects of MAPK inhibitors, PD98059 and SB203580, on the inhibition of phosphate uptake in OK cells by FGF-23. PD98059 and SB203580 are specific inhibitors of the upstream kinases of ERK and p38 MAPK (13, 14). PD98059 and SB203580 were confirmed to inhibit the phosphorylation of ERK and p38 MAPK in OK cells induced by FGF-23 (Fig. 7). PD98059 and SB203580 also completely blocked the inhibition of phosphate uptake by FGF-23, indicating the involvement of the Ras/MAPK pathway in the inhibition of the phosphate uptake by FGF-23 (Fig. 6).

**DISCUSSION**

Administration of FGF-23 decreased serum phosphate level in mice (3). Conditioned medium containing FGF-23 inhibited sodium-dependent phosphate uptake in OK cells (15). These results indicated that FGF-23 lowers serum phosphorus concentration by inhibition of phosphate reabsorption in renal epithelial cells. However, purified recombinant FGF-23 could not inhibit the phosphate uptake in OK cells (3). Therefore, these results indicated that FGF-23 might require other molecule(s) for the activity or further processing to the biologically active form. We prepared purified recombinant FGF-23 produced by the baculovirus expression system and examined the activity of FGF-23. As expected, FGF-23 alone could not inhibit phosphate uptake in OK cells. However, FGF-23 with heparin was found to significantly inhibit phosphate uptake, indicating that heparin-like molecules are essential for the activity of FGF-23. In contrast, FGF-2 could not inhibit phosphate uptake; the inhibitory activity was specific for FGF-23. FGF-23 rapidly (within ~1 h) inhibited phosphate uptake in OK cells, and then the inhibition increased gradually thereafter. The phosphate uptake in OK cells was shown to be mainly mediated by the type II Na/Pi cotransporter (8). However, the levels of the type II Na/Pi cotransporter mRNA were essentially unchanged during the rapid inhibition (~3 h), indicating that the inhibition of phosphate uptake by FGF-23 was regulated at the posttranscriptional level. PTH is a hormone that regulates the plasma levels of phosphate by inhibition of the reabsorption of phosphate in renal proximal tubules. PTH also rapidly inhibited phosphate uptake in OK cells. PTH inhibited phosphate uptake by leading to removal of the type II Na/Pi cotransporter from the apical membrane and to its subsequent degradation (17). Therefore, the rapid inhibition by FGF-23 was also expected to be regulated by removal of the type II Na/Pi cotransporter from the apical membrane.

PTH binds to PTH receptors on the surface membranes of target cells. The occupied receptors interact with guanyl nucleotide-regulated membrane-bound proteins that in turn activate membrane-bound adenyl cyclase to convert ATP to cyclic adenosine monophosphate (cAMP). cAMP is one of the intracellular second messengers that may be responsible for the action of PTH (18). In contrast phosphatonin, FGF-23, was shown not to increase cAMP formation in renal epithelial cells, indicating that phosphatonin, FGF-23, inhibited phosphate transport via a cAMP-independent mechanism (20). However, the mechanism of action remains to be elucidated.

FGFs exert their activity by binding to FGFs (12). There are four types of FGF gene, FGF-R1 to FGF-R4. Although the rat kidney expresses all four FGFs, renal proximal epithelial cells were shown to preferentially express FGF-R3 (10). By alternative splicing, two isoforms of FGF-3, FGF-3b and FGF-3c, could be generated (9). Therefore, we examined the isoforms of FGF-3 expressed in OK cells. The major isoform of FGF-3 expressed in OK cells was found to be FGF-3c. We also examined the binding of FGF-23 to the extracellular domain of FGF-3c by the Biacore system in the absence of heparin. Our results indicated that FGF-23 bound to the extracellular domain of FGF-3c with high affinity. Therefore, we expected that FGF-23 exerted its activity by binding to FGF-3c expressed in OK cells. FGF-23 could bind to FGF-3c in the absence of heparin. However, FGF-23 could inhibit phosphate uptake in the presence of heparin but not in the absence of heparin. Therefore, heparin-like molecules appear to be essential for activation of FGF-3c by FGF-23 but not for FGF-23 binding to FGF-3c.

FGFRs are receptor tyrosine kinases that have intrinsic protein tyrosine kinase activity and elicit tyrosine autophosphorylation of the receptors (12). An inhibitor specific for protein kinases of FGFs, SU 5402, blocked the inhibitory activity of FGF-23. These findings indicated that FGF-23 inhibited the phosphate uptake by activation of FGF-R3c. The major intracellular signaling pathway of FGF-23 was shown to be the Ras/MAPK pathway (12, 19). The ERK pathway and the p38 pathway are major components of the MAPK pathway. The ERK pathway generally plays roles in cell proliferation and differentiation, while the p38 pathway plays roles in cellular stress, inflammation, apoptosis, and differentiation. FGF-23 was found to significantly phosphorylate both ERK and p38 MAPK. An inhibitor of ERK, PD98059, and an inhibitor of p38 MAPK, SB203580, were also found to block the phosphorylation of ERK and p38 MAPK and inhibition of phosphate uptake by FGF-23. These results indicated that FGF-23 inhibited phosphate uptake in OK cells by activation of the MAPK pathway.

FGF-2 was shown to bind to most of FGFRs including FGF-R3c (21). We also found that FGF-2 could bind to FGF-R3c by the Biacore system. However, FGF-2 could not inhibit phosphate uptake even in the presence of heparin. Furthermore, FGF-2 was found to significantly induce phosphorylation of ERK but not that of p38 MAPK. These results also indicated that activation of both the ERK pathway and p38 MAPK pathway by FGF-23 was essential for inhibition of phosphate uptake in OK cells.

FGFs are local signaling molecules. FGFs exert their activity by binding to FGFRs in an autocrine/paracrine manner (12). However, FGF-23 mRNA expression was not detected in the kidney (3, 4, 5). Therefore, FGF-23 might be a hormone-like-signaling molecule. Although a variety of benign mesenchymal tumors were shown to abundantly express FGF-23, FGF-23 was barely detectable in normal tissues (3, 4, 5). Therefore, the physiological significance of FGF-23 remains to be elucidated.

In summary, the present study showed that (i) heparin-like molecules are essential for the activity of FGF-23, (ii) FGF-23 could bind to and activate FGF-R3c expressed in renal proximal epithelial cells, and (iii) activation of FGFR and the MAPK pathway was essential for the inhibitory activity. The present findings have revealed a novel MAPK-dependent mechanism of the regulation of phosphate uptake by FGF signaling.

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