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Mutant FGF-23 responsible for autosomal dominant hypophosphatemic rickets is resistant to proteolytic cleavage and causes hypophosphatemia in vivo

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FGF-23 is involved in the pathogenesis of two similar hypophosphatemic diseases, autosomal dominant hypophosphatemic rickets/osteomalacia (ADHR) and tumor-induced osteomalacia (TIO). We have shown that the overproduction of FGF-23 by tumors causes TIO. In contrast, ADHR derives from missense mutations in FGF-23 gene. However, it has been unclear how those mutations affect phosphate metabolism. Therefore, we produced mutant as well as wild-type FGF-23 proteins and examined their biological activity. Western blot analysis using site-specific antibodies showed that wild-type FGF-23 secreted into conditioned media was partially cleaved between Arg^{179} and Ser^{180}. In addition, further processing of the cleaved N-terminal portion was observed. In contrast, mutant FGF-23 proteins found in ADHR were resistant to the cleavage. In order to clarify which molecule has the biological activity to induce hypophosphatemia, we separated full-length protein, the N-terminal and C-terminal fragments of wild-type FGF-23. When the activity of each fraction was examined in vivo, only the full-length FGF-23 decreased serum phosphate. Mutant FGF-23 protein that was resistant to the cleavage also retained the activity to induce hypophosphatemia. The extent of hypophosphatemia induced by the single administration of either wild-type or the mutant full-length FGF-23 protein was similar. In addition, implantation of CHO cells expressing the mutant FGF-23 protein caused hypophosphatemia and the decrease of bone mineral content. We conclude that ADHR is caused by hypophosphatemic action of mutant full-length FGF-23 proteins that are resistant to the cleavage between Arg^{179} and Ser^{180}.

Hypophosphatemia with phosphaturia and inappropriately low serum 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] are characteristic of three related diseases, X-linked hypophosphatemic rickets/osteomalacia (XLH), autosomal dominant hypophosphatemic rickets/osteomalacia (ADHR) and a paraneoplastic syndrome called tumor-induced osteomalacia (TIO) (1). Because of some evidence that indicates humoral mechanism for the pathogenesis of XLH, a putative circulating phosphaturic factor named 'phosphatonin' has been proposed to be responsible for XLH and TIO (2, 3). On the other hand, the responsible gene for XLH was identified by positional cloning and termed phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX) (4). The protein encoded by this gene is a type II membrane integral protein composed of 749 amino acids. The structural homology and sequence similarity of PHEX to other zinc metallopeptidases such as neprilysin and endothelin-converting enzyme strongly suggest that PHEX has a peptidase activity (5). Therefore, an intriguing hypothesis that PHEX is a critical enzyme to degrade phosphatonin and XLH is caused by excess activity of phosphatnin because of inactivating mutations of PHEX has been proposed (2). However, physiological functions of PHEX protein and the identity of phosphatonin have been unclear.

TIO is a rare paraneoplastic disorder. Once the responsible tumor is identified and removed, abnormal metabolism for both phosphate and vitamin D rapidly disappears. In the previous study, we have cloned FGF-23 as a causative factor of TIO and demonstrated that FGF-23 causes hypophosphatemia, phosphaturia, osteomalacia and decreased $1,25(OH)_2D$ level in vivo (6). FGF-23 was first cloned in mouse as a new member of FGF family by homology to FGF-15 (7). It also has been identified as a responsible gene for ADHR (8). Therefore, FGF-23 is involved in the pathogenesis of at least two related diseases, TIO and ADHR.

In order to investigate molecular mechanisms by which missense mutations found in ADHR cause hypophosphatemia, we prepared wild-type and mutant recombinant FGF-23 proteins. In this study, we demonstrate that wild-type FGF-23 protein is cleaved at a specific site and only full-length FGF-23 has an activity to induce hypophosphatemia. We also show that mutations found in ADHR prevent the cleavage of FGF-23 and a cleavage-resistant mutant FGF-23 protein retains the activity to induce hypophosphatemia.

Abbreviations: ADHR, autosomal dominant hypophosphatemic rickets/osteomalacia; FE, fractional excretion; FGF, fibroblast growth factor; PHEX, phosphate-regulating gene with homologies to endopeptidases on the X chromosome; Pi, inorganic phosphate; TIO, tumor-induced osteomalacia; XLH, X-linked hypophosphatemic rickets/osteomalacia.

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Materials and Methods

Cell cultures

A cell line of Chinese hamster ovary cells stably expressing FGF-23 (CHO-FGF23) was established as described (6). CHO-FGF23 cells and PEAKrapid cells (Edge Biosystems, MD) were grown in alpha modified MEM supplemented with 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin (Life Technologies, MD). To prepare conditioned media, cells were washed once with PBS and cultured in serum-free SFM-II medium (Life Technologies, MD). Cultures were maintained at 37℃ in a humidified atmosphere of 5% CO_2/95% air.

Expression of recombinant proteins

The cDNA coding FGF-23 with His6 sequence at the C-terminus was prepared as described (6). Expression vectors for mutant FGF-23 were synthesized by in vitro mutagenesis using pEAK8 plasmid (Edge Biosystems, MD). Primers used were as follows; R176Q primers (Forward: 5'-CCCATACCACGGCAGCACACCCGGAG-3', Reverse: 5'-CTCCGGGTGTGCTGCCGTGGTATGGG-3'), R179Q primers (Forward: 5'-CGGCGGCACACCCAGAGCGCCGAGGA-3', Reverse: 5'-TCCTCGGCGCTCTGGGTGTGCCGCCG-3'), R179W primers (Forward: 5'-CGGCGGCACACCTGGAGCGCCGAGG-3', Reverse: 5'-CCTCGGCGCTCCAGGTGTGCCGCCG-3'), R176Q, R179Q primers (Forward: 5'-ATACCACGGCAGCACACCCAGAG-

CGCCGAG-3', Reverse: 5'- CTCGGCGCTCTGGGTGTGCTGCC-GTGGTAT-3'). For transient expression, each plasmid was introduced to PEAKrapid cells using the calcium phosphate method. Cells were cultured for 48 h after transfection and media were harvested. To generate stable cell lines expressing FGF-23 (R176Q, R179Q) mutant protein, pEAK8 plasmid containing the mutant cDNA was introduced into CHO cells and then drug-resistant clones were picked up in the presence of 5 μg/ml puromycin (Sigma, MO).

Separation of recombinant proteins

Conditioned media from CHO-FGF23 cells were filtrated through 0.2 μm membrane (SuporCap, Pall Gelman Laboratory, MI), and then applied to SP-Sepharose FF (Amersham Pharmacia Biotech, Little Chalfont, UK). C-terminal polypeptide fragment of FGF-23 was collected from the flow-through fraction. The retained proteins were eluted with linear gradient of NaCl ranged from 0 to 0.7M. The mature full-length and N-terminal polypeptide fragments were sequentially collected at approximately 0.3 and 0.4 M NaCl. Purified proteins were concentrated into a buffer consisted of 5 mM HEPES and 0.1 M NaCl, pH6.9. Mutant FGF-23 proteins transiently expressed in PEAKrapid cells were purified in the same way.

Antibody generation

Peptides (P-48: RNSYHLQIHKNGHVDGAPHQC and P-148: GMNPPPYSQFLSRRNEC) corresponding to the sequence between Arg^{48} and Gln^{67} and between Gly^{148} and Glu^{163} with additional Cys at the C-terminus were synthesized, conjugated with bovine thyroglobulin and used for immunization of rabbits. Antiserum was collected after 8 times immunization with 2-weeks intervals. Peptides were immobilized on a support (SulfoLink; Pierce, IL) via the side chain of terminal Cys residue and anti-peptide antibodies were affinity-purified using the peptide-coupled gel.

Western blotting

Conditioned media or purified fractions were resolved by 10-20% gradient SDS-PAGE under reduced condition and electroblotted onto a PVDF membrane. The membrane was incubated with anti-His (C-term)-HRP antibody (INVITROGEN, CA) or with polyclonal antibodies described above followed by the incubation with HRP-conjugated anti-rabbit IgG antibody. Signals were detected by ECL system (Amersham Pharmacia Biotech, Little Chalfont, UK).

Analysis of tryptic fragments

Purified FGF-23(R176Q, R179Q) protein was separated on SDS-PAGE gel and stained with Coomassie Brilliant Blue (CBB). Each single band was separately excised from the gel and digested by trypsin (Promega, WI). Molecular mass analyses of tryptic peptides were performed by Matrix-assisted Laser Desorption/Ionization time-of-flight mass spectrometry (MALDI-TOF/MS) using a Voyager-DE/STR (Applied Biosystems, CA). The peptides were identified by comparison of the molecular weight determined by MALDI-TOF/MS and theoretical mass of peptides from FGF-23.

Animals and experimental designs

Purified recombinant protein was intraperitoneally administered to six-weeks old male Balb/c mice (SLC, Japan). To collect urine samples, mice were bred in metabolic cages for 24 hours. Blood samples were taken under anesthesia with diethyl ether. To prepare tumor-bearing mice, approximately 1 x 10^7 cells were subcutaneously implanted into both sides of backs of six-weeks old male BALB/c athymic nude mice (SLC, Japan). All animals received a commercial rodent diet (CE-2; CLEA, Tokyo, Japan) containing 1.1% phosphate and 1.0% calcium. Diets and tap water were provided *ad libitum* throughout the experiments. All experiments were reviewed and approved by the institutional animal care and use committee at the Pharmaceutical Research Laboratory, KIRIN BREWERY Co., Ltd.

Results

Proteolytic cleavage of recombinant FGF-23

When the recombinant FGF-23 protein with His-tag sequence at the C-terminus was expressed in CHO cells, two recombinant products containing C-terminus were observed and identified as a mature FGF-23 protein lacking signal sequence and a processed polypeptide with Ser^{180} at the N-terminus (6) (Fig. 1A). To further investigate the processing of the recombinant FGF-23 protein, we prepared polyclonal antibodies against the synthetic peptides corresponding to the partial sequence of FGF-23. Both P-48 and P-148 antibodies showed the presence of multiple N-terminal fragments in addition to full-length FGF-23 (Fig. 1A). There seemed to be further processing of N-terminal fragment of FGF-23 besides cleavage between Arg^{179} and Ser^{180}, because P-48 antibody recognized small processed peptides that were not detected by P-148 antibody.

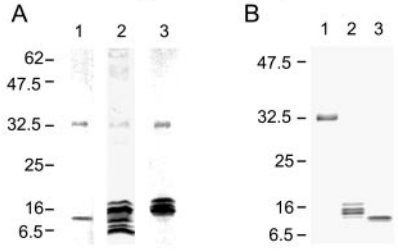

Figure 1. (A) Western blotting analysis of conditioned media from CHO-FGF23 cells using anti His-tag antibody (lane 1), P-48 antibody (lane 2) and P-148 antibody (lane 3). (B) Purified proteins by column chromatography were visualized by the Coomassie Brilliant Blue staining. Lane 1: full-length FGF-23, Lane 2: N-terminal fragments, Lane 3: C-terminal fragment.

Susceptibility of recombinant mutant FGF-23 proteins to the proteolytic cleavage

The preceding amino acid sequence of the cleavage site between Arg^{179} and Ser^{180}, Arg^{176}-His^{177}-Thr^{178}-Arg^{179}, is a consensus sequence of protease recognition motif, RXXR. All three missense mutations of FGF-23 gene in patients with ADHR, R176Q, R179Q and R179W, are in the consensus motif of RXXR (8). To investigate the implication of these mutations in the processing of FGF-23 proteins, several recombinant mutant FGF-23 proteins with substitutions of arginine residues in the RXXR motif were transiently expressed in PEAKrapid cells and analyzed by Western blotting. In contrast to the cleavage of wild-type FGF-23 protein between Arg^{179} and Ser^{180}, all three mutant proteins found in ADHR patients were secreted into conditioned media predominantly as a mature form (Fig. 2A, B). However, the mutant proteins appeared to be heterogeneous on SDS-PAGE. To clarify the cause of this heterogeneity by MALDI-TOF/MS, we generated a mutant FGF-23 protein with double mutations (R176Q, R179Q) that enabled to analyze tryptic fragments around the RXXR sequence by reducing the cleavage sites by trypsin. The recombinant protein with double mutations (R176Q, R179Q) also was resistant to the proteolytic processing between Arg^{179} and Ser^{180} and showed the similar heterogeneity to other mutant FGF-23 proteins (Fig. 2A, B). Four different protein bands around 32.5 kDa (Fig. 2C) were isolated, digested with trypsin and subjected to the mass spectrometric analysis. Results indicated that all four forms retained the full-length polypeptide sequence of FGF-23 (S^{25}-I^{251}) with variations of the number of o-linked sugar chains in the three tryptic fragments (162-175, 176-187, 199-228). All proteins possessed o-linked sugar chain in the 199-228 region. The variation of o-linked glycosylation occurred in the 162-175 region with one

attachment site and the 176-187 region with two attachment sites. The introduction of mutations probably influenced o-linked glycosylation to these attachment sites that are very close to the RXXR motif. These results indicate that mutations found in ADHR result in the resistance to the proteolytic cleavage between Arg^{179} and Ser^{180} by destroying the protease recognition consensus motif, RXXR.

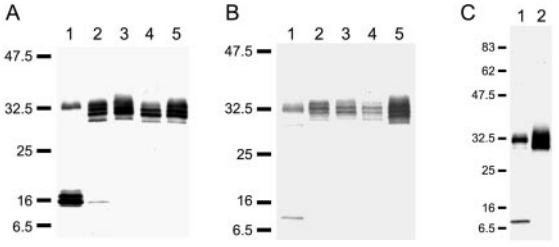

Figure 2. Analysis of mutant FGF-23 proteins by Western blotting. Transient expression of mutant FGF-23 proteins in PEAKrapid cells. Conditioned media were analyzed by Western blotting using P-148 antibody (A) and anti His-tag antibody (B). Lane 1: Wild-type FGF-23, Lane 2: FGF-23 (R176Q), Lane 3: FGF-23 (R179Q), Lane 4: FGF-23 (R179W), Lane 5: FGF-23 (R176Q, R179Q). (C) Conditioned media from a stable cell line expressing wild-type FGF-23 (lane 1) and double-mutant FGF-23 (R176Q, R179Q) (lane 2) were analyzed by Western blotting using anti His-tag antibody.

Biological activity of recombinant FGF-23 products

Since the clinical features of ADHR patients are similar to those of TIO, it is likely that these two diseases derive from a common mechanism of excessive biological activity of FGF-23. To investigate how the processing of the FGF-23 protein influences the biological activity, we first examined the activity of cleaved fragments. Proteins containing full-length mature FGF-23, N-terminal portion and C-terminal fragment of FGF-23 were separated by combination of ion-exchange and metal-affinity chromatography (Fig. 1B). These protein fractions were intraperitoneally administrated into mice twice with an interval of 12 hours. The significant decrease of serum phosphate and increase of renal phosphate excretion (FEPi) were observed only in mice administered mature FGF-23 protein (Table 1). N-terminal and C-terminal fragments of FGF-23 did not affect serum phosphate level or FEPi, either. Serum calcium level did not change by any treatment. These results indicate that the processing between Arg^{179} and Ser^{180} abolishes the activity of the FGF-23 protein to induce phosphaturia and hypophosphatemia.

Table 1. Biological activity of separated recombinant products

	sPi (mg/dl)	sCa (mg/dl)	FEPi
Vehicle	8.10 ± 0.33	9.01 ± 0.08	0.266 ± 0.200
Full length	5.83 ± 0.20**	9.01 ± 0.13	0.390 ± 0.028*
N-fragment	8.91 ± 0.17	9.25 ± 0.08	0.233 ± 0.014
C-fragment	8.94 ± 0.38	8.96 ± 0.13	0.257 ± 0.010

Each protein fraction was administered to mice twice (5 μg each) with an interval of 12 hours (n = 5). At 24 hour after the first administration, fractional excretion of phosphate (FEPi), serum levels of phosphate and calcium were measured. Results are expressed as means +/- SEM. *P<0.005 and **P<0.001 against vehicle by one-way ANOVA followed by Bonferroni's method for comparison of multiple means.

To clarify the relationship between the resistance to the cleavage and biological activity of FGF-23 more directly, we analyzed biological activity of the mutant proteins *in vivo*. Recombinant mutant proteins corresponding to the full-length mature form were purified and administered to mice three times with an interval of 6 hours. Mutant FGF-23 proteins also caused the decrease of serum phosphate level at 24 hours after the first injection to the similar extent to that induced by wild-type FGF-23 (Fig. 3). These results indicated that the mutant protein retained biological activity to induce hypophosphatemia.

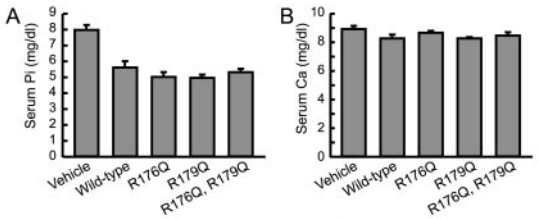

Figure 3. Biological activity of mutant FGF-23 proteins in vivo. Purified full-length mutant FGF-23 proteins (4 μg/infusion) were administered intraperitoneally into mice three times with an interval of 6 hours (n = 4). Blood was obtained at 24 hours after the first injection and serum phosphate (A) and calcium (B) levels were determined. Results are expressed as means +/- SEM.

To examine the long-term effects of mutant FGF-23 protein, we implanted the same number of CHO cells stably expressing the wild-type or mutant protein into athymic nude mice and allowed cells to form tumors. Hypophosphatemia and reduced bone mineral content were observed in both groups (Fig. 4). These results confirmed that the cleavage-resistant FGF-23 protein has an ability to cause hypophosphatemic bone disease.

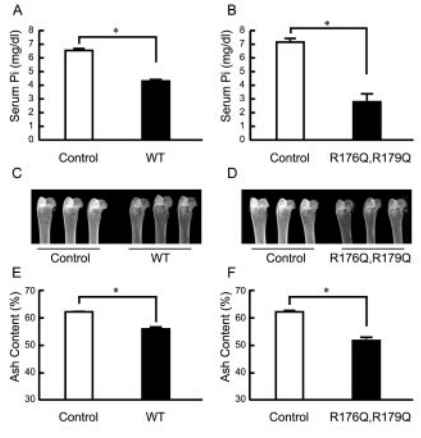

Figure 4. Serum phosphate and bone mineral content of mice with CHO cells stably expressing wild-type or mutant FGF-23(R176Q, R179Q). Wild-type CHO cells were implanted in each experiment as a control. After 44 days, blood samples and femurs were collected. (A) (B) Serum phosphate levels. (C) (D) X-ray images of femurs. (E) (F) The ratio of ash weight to dry weight of femurs (n=3). Results are expressed as means +/- SEM. *P<0.001 by Student's t-test.

Discussion

FGF-23 was identified as a gene responsible for ADHR (8). However, it has been unknown how mutations of FGF-23 cause ADHR. We have shown that overexpression of FGF-23 caused hypophosphatemia and osteomalacia, which are common features to both TIO and ADHR (6). Therefore, we speculated that the mutations of FGF-23 found in ADHR somehow enhance the activity of FGF-23 to induce hypophosphatemia. The proteolytic processing between Arg^{179} and Ser^{180} has been observed when recombinant FGF-23 was expressed in CHO cells (6). Detection of the cleaved C-terminal protein of FGF-23 has also been reported by other groups (9, 10). However, little has been known about the processing of residual N-terminal portions of FGF-23 protein.

In this study, we showed that wild-type FGF-23 is processed at multiple sites especially in N-terminal portion as shown in Fig.1 and most of these proteolytic cleavages occur only after the processing between Arg^{179} and Ser^{180}, because heterogeneity of mutant FGF-23 was derived from differences in the number of sugar chains (Fig. 2). In addition, we demonstrated that neither FGF-23 lacking the unique C-terminal domain nor the C-terminal fragment induced hypophosphatemia (Table 1). These results indicate that the cleavage between Arg^{179} and Ser^{180} is the primary processing of FGF-23 that converts biological active FGF-23 into inactive metabolites in terms of the induction of hypophosphatemia. Furthermore, we demonstrated that all types of mutations found in ADHR patients resulted in the resistance to the processing as described recently (14) and the cleavage-resistant mutations increased the ratio of full-length protein to its inactive metabolites (Fig. 2). We also demonstrated that the cleavage-resistant mutant full-length protein retained the activity to induce hypophosphatemia. These findings suggest that circulatory level of full-length active FGF-23 is increased in patients with ADHR even if expression of FGF-23 is not enhanced.

When the same amount of full-length wild-type and mutant FGF-23 proteins were injected into mice (Fig. 3), wild-type and mutant full-length FGF-23 proteins showed similar potency to induce hypophosphatemia. In contrast, hypophosphatemia and reduced bone mineral content of mice with CHO cells expressing the mutant FGF-23 tended to be more severe than those with wild-type FGF-23 (Fig. 4). This could be explained by higher circulatory level of full-length mutant FGF-23 protein that is resistant to the cleavage. However, it is impossible to directly compare the severity of effects of CHO cells expressing either wild-type or the mutant FGF-23 protein in this model because the expression level of FGF-23 proteins by CHO cell tumors can not be manipulated. Our results indicate that certain amount of either full-length wild-type or the cleavage-resistant mutant FGF-23 causes hypophosphatemic bone disease. Therefore, establishment of more sensitive in vitro assay for biological activities of FGF-23 and measurement of circulatory level of full-length FGF-23 are necessary to compare relative potency and in vivo stability of wild-type and mutant FGF-23 proteins more accurately.

The biological activity of FGF-23 is reminiscent of the putative phosphaturic factor in XLH. The hypothesis that a common phosphaturic factor of TIO and XLH is a substrate for PHEX has been proposed. Although the cleavage of FGF-23 at RXXR motif by PHEX was recently suggested using in vitro translated proteins (10), other sequences than RXXR were suggested as targets of PHEX (11, 12). Recent report also showed that PHEX could not degrade a peptide fragment of FGF-23 containing the RXXR motif (13). In addition, we observed the cleavage of FGF-23 in PEAKrapid cells in which expression of PHEX was not observed by RT-PCR (data not shown). The mutant FGF-23 proteins do not seem to be completely resistant to the cleavage, because small amount of cleaved fragment was still observed (Fig. 2). In addition, the presence of fragments with about 25 kDa was commonly observed as faint signal in lanes with mutant FGF-23 proteins when the exposure period was elongated (data not shown). Additional study is clearly necessary to clarify the relationship between PHEX and FGF-23.

In conclusion, the biological activity of full-length wild-type FGF-23 to induce hypophosphatemia is lost by cleavage between Arg^{179} and Ser^{180}. Mutations in the RXXR motif found in ADHR patients prevent the proteolytic cleavage, and the mutant FGF-23 protein that is resistant to the cleavage retains the activity to induce hypophosphatemia. Therefore, it is concluded that ADHR is caused by hypophosphatemic action of mutant full-length FGF-23 proteins.

Acknowledgments

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