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Posttranslational processing of FGF23 in osteocytes during the osteoblast to osteocyte transition

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ABSTRACT

FGF23 is an O-glycosylated circulating peptide hormone with a critical role in phosphate homeostasis; it is inactivated by cellular proprotein convertases in a pre-release degradative pathway. We have here examined the metabolism of FGF23 in a model bone cell line, IDG-SW3, prior to and following differentiation, as well as in regulated secretory cells. Labeling experiments showed that the majority of ^{35}S-labeled FGF23 was cleaved to smaller fragments which were constitutively secreted by all cell types. Intact FGF23 was much more efficiently stored in differentiated than in undifferentiated IDG-SW3 cells. The prohormone convertase PC2 has recently been implicated in FGF23 degradation; however, FGF23 was not targeted to forskolin-stimulatable secretory vesicles in a regulated cell line, suggesting that it lacks a targeting signal to PC2-containing compartments. *In vitro*, PC1/3 and PC2, but not furin, efficiently cleaved glycosylated FGF23; surprisingly, PC5/6 accomplished a small amount of conversion. FGF23 has recently been shown to be phosphorylated by the kinase FAM20C, a process which was shown to reduce FGF23 glycosylation and promote its cleavage; our *in vitro* data, however, show that phosphorylation does not directly impact cleavage, as both PC5/6 and furin were able to efficiently cleave unglycosylated, phosphorylated FGF23. Using qPCR, we found that the expression of FGF23 and PC5/6, but not PC2 or furin, increased substantially following osteoblast to osteocyte differentiation. Western blotting confirmed the large increase in PC5/6 expression upon differentiation. FGF23 has been linked to a variety of bone disorders ranging from autosomal dominant hypophosphatemic rickets to chronic kidney disease. A better understanding of the biosynthetic pathway of this hormone may lead to new treatments for these diseases.

1. Introduction

The circulating peptide hormone FGF23, a known regulator of bone mineralization and serum phosphate levels, was first described in brain [1] but is most highly expressed and released from immature and mature osteocytes within the bone matrix [2,3]. While a great deal of information is available on the physiology of this important peptide hormone and its effects on the kidney, heart, and other organs [4], detailed information as to the biosynthesis and storage of osteocyte FGF23 is still lacking. Many studies (performed in HEK and undifferentiated osteoblast cell lines) have shown that the release of FGF23 as an intact bioactive entity is controlled by intracellular proteolytic degradative processing (reviewed in [5]). Cleavage destroys the ability of FGF23 to act on the kidney FGFR1 receptor (reviewed in [4]), and the cellular cleavage process is closely controlled to ensure the release of appropriate amounts of intact FGF23. Human diseases in which intact circulating FGF23 is raised result in hypophosphatemia, while diseases in which intact FGF23 is lowered result in hyperphosphatemia (reviewed in [4,5]). O-glycosylation of FGF23 by the enzyme GalNT3 is required for the secretion of intact protein by blocking its degradation [6–9]. More recently, FGF23 has been shown to be the target of phosphorylation by the new secretory kinase FAM20C [10,11]; while phosphorylation and O-glycosylation represent competitive processes [10], the intracellular fate of phosphorylated FGF23 is not yet clear.

The degradative cleavage of FGF23 is thought to be accomplished by a member of the proprotein convertase family, since it is blocked by general convertase inhibitors such as decanoyl RVKR-chloromethyl ketone [12,13], and known human mutations within the convertase cleavage site which remove the convertase consensus site decrease

Abbreviations: ER, endoplasmic reticulum; CMK, decanoyl RVRR-chloromethyl ketone; PC2, prohormone convertase 2; PC5/6, proprotein convertase 5/6; TGN, trans-Golgi network.

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cellular degradative cleavage and enhance release of intact FGF23, resulting in severe bone disease [8,14]; reviewed in [4]. The mammalian eukaryotic proprotein convertases (reviewed in [15,16]) constitute a family of serine proteases usually associated with the biosynthesis of secreted proteins; the degradative cleavage of FGF23 is unusual in this regard. Precisely which convertases are involved in FGF23 cleavage is not yet clear, and recent work has implicated both furin [10] and the prohormone convertase PC2 (together with its obligate binding partner 7B2; [17]) in FGF23 degradation [18]. However, whereas furin activity is associated with the constitutive secretory pathway, both proPC2 maturation as well as PC2 enzymatic activity require the acidic environment present within the regulated secretory pathway (pH 5; reviewed in [19]). While regulated secretory granules within endocrine cells undergo acidification upon maturation, providing the appropriately acidic compartment, it is not clear whether osteocytes contain an analogous secretory compartment and/or whether FGF23 itself contains granule sorting information which would direct it to such a compartment. We address this latter question in the study below.

A major problem in studying the osteocyte as an endocrine cell is the fact that osteocytes are quite difficult to access. A model system has been developed to study osteocyte differentiation in which cell proliferation is induced by a thermolabile SV40 large-T antigen regulated by γ-interferon [20]. When these cells, termed IDG-SW3, are cultured at 37 °C in the absence of γ-interferon, T-antigen expression is dramatically reduced within 24 h and the cells differentiate, faithfully reproducing primary osteoblast to osteocyte differentiation [20]. Differentiated IDG-SW3 cells produce and mineralize an extracellular matrix, and display many of the hallmark proteins of differentiated osteocytes, such as E11/gp38, Dmp1, Phex, Mepe, and sclerostin, in addition to increased FGF23.

In the report presented below, we have investigated the biochemistry and cell biology of FGF23 cleavage, and the potential contribution of various convertases as well as of the secretory chaperone 7B2, in both undifferentiated and differentiated osteocyte model cells. In order to compare osteocyte FGF23 processing to that occurring in known secretory processing systems containing PC2, FGF23 synthesis was also examined in an established regulated secretory model cell line, AtT-20/PC2 [21].

2. Materials and methods

2.1. Materials

The preparation of recombinant mouse PC1/3, PC2, and soluble human furin from Chinese hamster ovary cell-conditioned medium has been described previously [22–24]. The purity of these recombinant enzymes was estimated at greater than 95% using SDS-PAGE stained with Coomassie blue. Recombinant human PC5/6A, produced in S2 cells, was obtained from Robert Day, University of Sherbrooke [25], and soluble human PC7 (Leu38-Thr667, His-tagged at the amino terminus) was purchased from R&D Systems, Minneapolis, MN (2984-SE-010). The Flag-tagged human FGF23 vector and the GalNT3 vector were generous gifts of Shoji Ichikawa and Michael Econs, University of Indiana [8], while the pCAGEN vector used for subcloning human FGF23 was obtained from Joseph Stains (University of Maryland-Baltimore). The Flag-tagged vectors encoding FAM20C and its inactive D478A variant were obtained from Vincent Tagliabracci and Jack Dixon [26]. Recombinant bacterial hFGF23 was obtained from PeproTech (Rocky Hill, NJ), while the glycosylated hFGF23 (prepared via eukaryotic expression and lectin purification and C-terminally His-tagged) was obtained from R&D Systems. AtT-20 cells stably expressing mouse PC2 were obtained from Richard Mains, University of Connecticut Health Sciences Center [21].

2.2. Methods

In vitro proteolysis reactions using recombinant proprotein convertases—Recombinant C-terminally His-tagged glycosylated human FGF23 (1 μg of lectin-purified material, purchased from R&D Systems, Minneapolis, MN, 2604-FG-025/CF) was incubated with 5 units of either furin, PC1/3, PC2, PC5/6, or PC7 in 50 μl reactions containing appropriate enzyme assay buffers (100 mM sodium acetate, 5 mM $CaCl_2$, 0.05% Brij-35 pH 5.5 for PC1/3; 100 mM sodium acetate, 5 mM $CaCl_2$, 0.05% Brij-35 pH 5.0 for PC2; 100 mM HEPES, 5 mM $CaCl_2$, 0.05% Brij-35 pH 7.0 for furin; 20 mM bis-Tris, 1 mM $CaCl_2$, 0.1% Triton pH 6.5 for PC5/6; 50 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij-35 pH 7.5 for PC7) at 37 °C for 100 min. The reactions were then subjected to SDS-PAGE using 15% Tris–HCl acrylamide gels. Cleavage patterns were analyzed by Western blotting using the R&D polyclonal FGF23 antibody (AF2604, 1:1000). One unit of PC activity is equal to the amount of the enzyme required to cleave 1 pmol/min of pRTKR-aminomethyl coumarin fluorogenic substrate (Peptides International, Louisville, KY).

In other experiments, 2 μg of recombinant human FGF23 lacking all posttranslational modifications (generated in bacteria) was incubated with either Flag-tagged $FAM20C^{WT}$ or $FAM20C^{D478A}$ immunopurified from transiently-transfected HEK cell medium using anti-Flag resin together with 5 μCi of γ-labeled ATP (10 Ci/mmol, Perkin Elmer) in kinase assay buffer (100 mM HEPES pH 7.0, 10 mM $MnCl_2$, 100 mM NaCl, 1 mg/ml BSA) at 30 °C for 1 h prior to cleavage with 200 ng recombinant human soluble furin or PC5/6 in its specific assay buffer (see above). Reactions were separated on 18% SDS-PAGE gels and analyzed both by Western blotting using the polyclonal R&D FGF23 antiserum (AF2604, 1:1000) as well as by phosphoimaging to assess transfer of radioactive phosphate. These Western blots were cross-linked in 3% glutaraldehyde in PBS for 30 min prior to blocking with milk in order to better retain the C-terminal peptide.

IDG-SW3 cell culture—The details of IDG-SW3 cell culture have already been described [20]. Tissue culture medium (α-MEM), fetal bovine serum, and recombinant mouse γ-interferon (IFN-γ, PMC4031) were purchased from Gibco Life Technologies (Grand Island, NY). Rat tail collagen type 1, 99% pure, was purchased from BD Biosciences (Bedford, MA, USA). Cells were expanded under growth conditions (33 °C) in α-MEM with 10% heat-treated FBS and 50 U/ml of gamma interferon (IFN-γ) on rat tail type 1 collagen-coated flasks. To induce osteogenesis, confluent SW3-FGF23 cells were exposed to osteogenic conditions (37 °C, in α-MEM medium containing 10% heat-treated FBS and 50 μg/ml of ascorbic acid and 4 mM β-glycerophosphate (Sigma, St. Louis, MO) but lacking IFN-γ; "osteogenic medium"). Both undifferentiated and differentiated cells were plated into 6-well collagen-coated plates for transfection, blotting, and radioimmunoassay experiments. Cells were differentiated to the desired time points, replacing osteogenic medium every 3–4 days.

Generation of an FGF23-expressing cell line (*SW3-FGF23*)—SW3 cells maintained under growth conditions were split from a confluent T-25 flask to three 10-cm collagen-coated dishes and two dishes were transfected with 10 μg each of N-terminal Flag-tagged human FGF23 [27] using Fugene HD overnight at 33 °C. The medium was replaced twice a week with medium containing 250 μg/ml G418 until colonies began to form. Clones were screened for FGF23 secretion by Western blotting and the highest expressing clone chosen. However, expression of FGF23 was greatly diminished after two to three passages, indicating rapid loss of the transgene. For this reason, these cells were always additionally transiently transfected with FGF23 cDNA prior to experimentation. On some occasions, SW3-FGF23 cells were used for transient transfection with FGF23 cDNA. No differences were detected between cells which were transiently transfected and SW3-FGF23 cells, supporting the rapid loss of transgene expression.

Transient transfection—As described above, in order to obtain increased expression of FGF23, we transiently transfected SW3 cells with a Flag-tagged FGF23 vector prior to labeling. The Flag-tagged insert from pcDNA3-FGF23 [8] was first subcloned into the pCAGEN vector at the *EcoR1* and *Xho1* sites. SW3 and SW3-FGF23 cells grown as described above were transfected with 2 μg per well of pCAGEN-FGF23 cDNA; transfections were also performed using 1 μg of FGF23 cDNA in the presence or absence of 1 μg of vectors encoding rat 27 kDa 7B2 [17] and/or murine proPC2 [28] using Fugene HD. Total cDNA was kept constant