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FGF23 induces expression of two isoforms of NAB2, which are corepressors of Egr-1

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Abstract

Fibroblast growth factor 23 (FGF23) is a key humoral factor in phosphate homeostasis and skeletogenesis, though the nature of its intracellular signaling is still unclear. Recently, Egr-1, a zinc-finger transcription factor, was identified as an immediate early response gene of FGF23 in the kidney. We report here, that FGF23 induces not only Egr-1 but also two isoforms of NAB2, which are specific co-repressors of Egr-1. Both isoforms of NAB2 induced by FGF23 were localized in the nucleus and suppressed the transcriptional activity of Egr-1. A negative feedback loop established by Egr-1 and NAB2 may thus be involved in mediating the physiological effects of FGF23.

Keywords: FGF23; Egr-1; NAB2; Co-repressor; Transcription

Fibroblast growth factor 23 (FGF23) is a member of the FGF family and a key humoral factor in phosphate homeostasis and skeletogenesis [1–4]. Elevation of serum levels of FGF23 causes hypophosphatemia and skeletal abnormalities in patients who have mutations of the FGF23 gene or tumor cells over-expressing FGF23, such as those with autosomal-dominant hypophosphatemic rickets, tumor-induced osteomalacia, and X-linked hypophosphatemia [5–7]. FGF23 activity is regulated by proteases and its specific receptors. Mature FGF23 is degraded to two small fragments by furin family proteases [8]. Recently, Klotho and FGF type I receptors were identified as binding and signaling receptors, respectively [9,10]. Although FGF type I receptors are widely expressed in various tissues, systemically administered FGF23 acts only in the kidney [10]. Klotho, which is abundantly expressed in the kidney, appears to be a specific regulator of the activation of FGF23 signaling [10]. However, the intracellular signaling of FGF23 through these receptors remains to be clarified.

Early growth response-1 (Egr-1), also termed nerve growth factor-induced clone A (NGFI-A) or Krox-24, is an immediate early response transcription factor with zinc-finger motifs [11–13]. Egr-1 couples extracellular signals to the induction of cellular programs for differentiation, growth, and cell death [11,14]. Transcription of Egr-1 has been observed during osteoblast differentiation and tooth development, suggesting that Egr-1 plays important roles in bone and teeth [15,16].

Abbreviations: FGF, fibroblast growth factor; Egr-1, early growth response-1; NGFI-A, nerve growth factor-induced clone A; NAB, NGFI-A binding; NCD, NAB conserved domain.

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The transcriptional activity of Egr-1 is negatively regulated by constitutive and inducible co-repressors, NGFI-A-binding protein (NAB) 1 and 2, respectively [13,17–21], which have highly conserved regions in their N-terminal and C-terminal portions termed NAB conserved domains (NCD) 1 and 2, respectively. It has been proposed that NCD1 is required for both interaction with Egr-1 and homo-oligomerization [11], and that NCD2 is essential for transcriptional repression [22,23]. The repressive function of NAB2 is mediated through two independent domains. The chromodomain helicase DNA-binding protein 4 interacting domain (CID) is recruited into the nucleosome remodeling and deacetylase complex through direct interaction. NCD2 is an independent repressive domain of NAB2 that exerts its repressive function through unidentified HDAC-independent mechanisms [24]. In contrast to full-length NAB2, an alternative form lacking exon 3 (NAB2ΔE3) lost repressive capacity due to disruption of NCD2 and a nuclear localization signal [12]. Egr-1 directly activates the promoter activity of NAB2 and thereby establishes a negative feedback loop to suppress extracellular signaling [25].

Recently, Egr-1 was identified as one of the immediate early target genes of FGF23 signaling in the kidney *in vivo* [10]. This finding suggested that an Egr-1-dependent pathway plays an important role in intracellular signaling through FGF23 receptors. In the present study, we found that FGF23 induced not only Egr-1 but also two isoforms of NAB2, a full-length form and one lacking exon 6 (NAB2ΔE6). In contrast to NAB2ΔE3, NAB2ΔE6 exhibited sustained capacity to suppress the transcriptional activity of Egr-1 as a full-length form of NAB2. Taken together, these findings suggest that the negative feedback loop established by Egr-1 and NAB2 may play roles in mediating the physiological effects of FGF23 in phosphate homeostasis and skeletogenesis.

Materials and methods

Administration of FGF-23 in vivo and in vitro. Balb/c mice at 6 weeks of age were i.p. injected with FGF23 at 20 μg/head. After 0.5 h, the kidney was removed under anesthesia and total RNA was extracted. HEK293T cells were treated with conditioned medium containing human FGF23 prepared by transient transfection of an expression vector carrying human FGF23 with R176Q and R179Q mutations in COS7 cells. Concentrations of FGF23 in conditioned medium were determined with a FGF23 ELISA kit (KAINOS, Tokyo, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed using total RNA as described [26]. The primers used were as follows: human NAB2, 5′-tggccgtttcgactctaagc-3′ (forward) and 5′-caggacatctgtccagcagc-3′ (reverse), mouse NAB2, 5′-gacgatcatgacgcccag-3′ (forward) and 5′-cttcccccactttcactgcc-3′ (reverse), human Egr-1, 5′-gacagcagtcccatttactc-3′ (forward) and 5′-atcttggtatgcctcttgcg-3′ (reverse), mouse Egr-1, 5′-cttaataccacctactaccaatcccagc-3′ (forward) and 5′-gttgaggtgctgaaggagctgctga-3′ (reverse).

Co-immunoprecipitation and immunoblotting. For immunoprecipitation, cells were co-transfected with expression vectors for FLAG-mouse Egr-1 and Myc-mouse NAB2 proteins using Lipofectoamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 20 h, the cells were lysed in buffer [10 mM Tris–HCl (pH 7.5), 1% Triton X-100, 0.15 M NaCl, 1× protease inhibitor cocktail] and supernatants of the extracts were immunoprecipitated with anti-FLAG M2 agarose beads (Sigma–Aldrich, St. Louis, MO). After repeated washing with the lysis buffer, precipitates were subjected to immunoblotting using anti-Myc antibody (clone 9E10, Santa Cruz Biotechnology, Santa Cruz, CA).

Immunohistochemical staining. Immunohistochemical staining was performed as described. In brief, cells co-transfected with expression vectors for FLAG- and His-tagged proteins were fixed with 10% formalin 18 h later and doubly stained with anti-FLAG (clone M2, Sigma–Aldrich) and anti-Myc (MBL Medical & Biological Laboratories Co., Nagoya, Japan) antibodies.

Luciferase activity. Cells were co-transfected with expression vectors for Egr-1 and NAB2 with Id985-luc carrying a Egr-1 consensus sequence and pRL-SV40 (Promega, Madison, WI) using Lipofectamine 2000 (Invitrogen) as described previously [27]. After 20 h, luciferase activity was determined using a Dual Luciferase assay kit (Promega).

Results

FGF23 induces expression of NAB2 in vivo and in vitro

Recently, Egr-1 was identified as an immediate early response gene of FGF23 in the kidney [10]. We found that administration of FGF23 stimulated two transcripts of NAB2 of different sizes in the mouse kidney within 0.5 h (Fig. 1A). Stimulation of these NAB2 transcripts by FGF23 was also detected at 2 h in HEK293T cells with over-expression of exogenous Klotho (Fig. 1B). In these cells, Egr-1 mRNA was also induced within 0.5 h by FGF23 (Fig. 1B). Determination of DNA sequences of the long and short transcripts of NAB2 indicated that they were the full-length (NAB2full) and an alternatively spliced form of NAB2 lacking exon 6 (NAB2ΔE6), respectively (Fig. 1C). The transcripts of both NAB2full and NAB2ΔE6 were ubiquitously expressed in various tissues in mice (Fig. 1D). We detected no isoform of NAB2 lacking exon 3 (NABΔE3) in any types of cells examined (data not shown).

Cellular localization and functions of two types of NAB2 isoforms

Immunoprecipitation followed by Western blot analysis indicated that both NAB2full and NAB2ΔE6 interacted with Egr-1 and formed homo- and hetero-oligomers with each other (Fig. 2A). However, neither NAB2full nor NAB2ΔE6 exhibited capacity to interact with osterix, another type of C2H2 zinc-finger protein (Fig. 2A). Transient over-expression of Myc-tagged NAB2full or NAB2ΔE6 with FLAG-tagged Egr-1 indicated that all of these proteins were localized in the nucleus, suggesting that they had formed complexes in the nuclei (Fig. 2B). Both NAB2full and NAB2ΔE6 suppressed the transcriptional activity of Egr-1 (Fig. 2C).

A previous study revealed that a point mutation of E51K in NCD1 in mouse NAB1 interfered with its ability to bind to Egr-1 [17]. We therefore introduced the corresponding mutation, E82K, into mouse NAB2full and

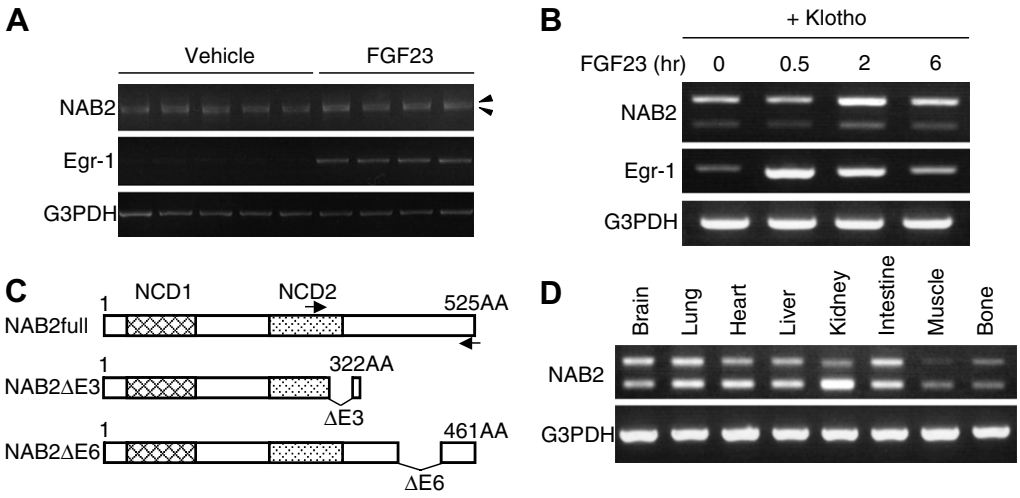

Fig. 1. FGF-23 induced NAB2 expression. (A) FGF23 induced expression of Egr-1 and NAB2 in mouse kidney. Mice were injected i.p. with FGF23 at 20 μg/head, and then total RNA was prepared from the kidney after 0.5 h. RT-PCR analysis was performed for Egr-1, NAB2, and G3PDH. (B) FGF23 induced transcription of both Egr-1 and NAB2 *in vitro*. HEK293T cells with Klotho over-expression were treated with conditioned medium containing FGF23 for the indicated period. RT-PCR analysis was performed for Egr-1, NAB2, and G3PDH. (C) Schematic structures of three isoforms of NAB2, NAB2full, NAB2ΔE3, and NAB2ΔE6. Two independent, highly conserved domains in NAB proteins are indicated as NCD1 and NCD2. Primers used in RT-PCR analysis are indicated by arrows. (D) RT-PCR analysis of NAB2 mRNAs in various tissues of mice.

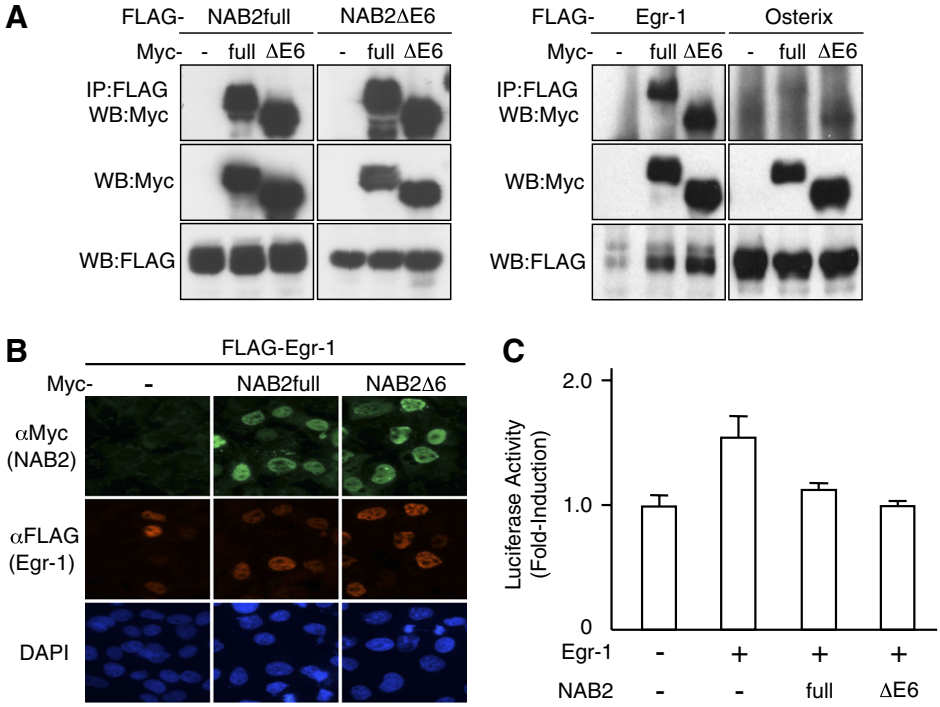

Fig. 2. Comparison of properties of NAB2full and NAB2ΔE6. (A) Interactions of NABfull and NAB2ΔE6 and Egr-1. FLAG-tagged NAB2full, NAB2ΔE6, Egr-1 or Osterix and Myc-tagged NAB2full or NAB2ΔE6 were co-transfected in COS7 cells, and then immunoprecipitated with anti-FLAG M2 agarose beads. The precipitates were subjected to Western blot analysis using anti-Myc antibody. (B) Cellular location of NABfull or NAB2△E6 and Egr-1. Myc-tagged NAB2full or NAB2ΔE6 was co-transfected with FLAG-tagged Egr-1 in HEK293T cells. The cells were immunohistochemically stained with anti-FLAG and anti-Myc antibodies. (C) NAB2full and NAB2ΔE6 suppressed the transcriptional activity of Egr-1. A luciferase reporter carrying an Egr-1 consensus sequence was co-transfected with NAB2full or NAB2△E6 in the presence of Egr-1. Luciferase activities were determined using a kit.

NAB2ΔE6 and examined their properties. The E82K mutation abolished the abilities of both NAB2full and NAB2ΔE6 to bind to Egr-1, though they were still able to form homo- and hetero-oligomers with each other (Fig. 3A). Immunohistochemical analysis indicated that these E82K mutants of NAB2, especially NAB2△E6(E82K), formed snake-like structures in the nuclei, although Egr-1 was distributed homogeneously in

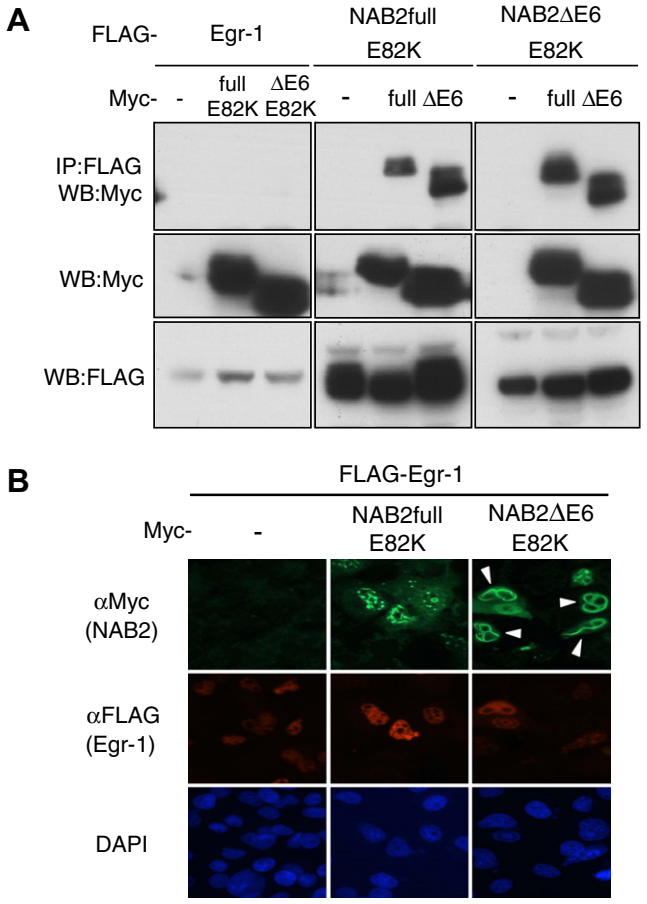

Fig. 3. Characterization of properties of NAB2full and NAB2ΔE6 with E82K mutation. (A) Interactions of NABfull(E82K) and NAB2-ΔE6(E82K) with Egr-1. FLAG-tagged Egr-1 and Myc-tagged NAB2full(E82K) or NAB2△E6(E82K) were co-transfected in COS7 cells, and then immunoprecipitated with anti-FLAG M2 agarose beads. The precipitates were subjected to Western blot analysis using anti-Myc antibody. (B) Cellular location of NABfull(E82K) or NAB2ΔE6(E82K) and Egr-1. Myc-tagged NAB2full(E82K) or NAB2ΔE6(E82K) was co-transfected with FLAG-tagged Egr-1 in HEK293T cells. The cells were immunohistochemically stained with anti-FLAG and anti-Myc antibodies. Typical snake-like structures are indicated by the arrowheads.

the nucleus (Fig. 3B). Thus, the location of the E82K mutant proteins of NAB2 differed completely from that of Egr-1.

Discussion

FGF23 is an important factor in phosphate homeostasis and skeletogenesis in vertebrates [2–4]. Although the molecular mechanisms in FGF23 signaling have not been elucidated, Klotho and FGF type I receptors were recently identified as specific receptors for FGF23 [9,10]. In addition, recent work revealed that FGF23 induced Egr-1 expression as an immediate early response gene in the kidney *in vivo* [10]. In the present study, we found that FGF23 induced expression of not only Egr-1 but also NAB2 *in vivo* and *in vitro* in the presence of Klotho. Mice doubly deficient in NAB1 and NAB2 exhibited abnormalities in various tissues, including the skeleton [28]. It has been reported that Egr-1 directly activates the promoter activity of NAB2 [25]. Taken together, these findings suggested that FGF23 induces Egr-1 and then NAB2 in a negative feedback loop to suppress its own signaling. Egr-1- and NAB2-dependent intracellular signaling may play roles in mediating the physiological effects of FGF23, such as phosphate homeostasis and skeletogenesis. This possibility can be tested by administration of FGF23 to Egr-1 or NAB2-deficient mice in the future.

We found that FGF23 induced two isoforms of NAB2 transcripts, a full-length form and an alternative spliced one lacking exon 6, NAB2ΔE6. Another form of NAB2 lacking exon 3, NAB2ΔE3, had been detected in the cytoplasm and had lost the ability to inhibit the transcriptional activity of Egr-1 due to disruption of NCD2 and nuclear localization signal [12]. The present findings suggest that both NAB2full and NAB2ΔE6 are major forms of NAB2 in various tissues and cell lines. In contrast to NAB2ΔE3, however, NAB2ΔE6 had both complete NCD2 and nuclear localization domain and had retained the ability to bind to Egr-1 and suppress its transcriptional activity. These findings clearly indicated that NAB2ΔE6 is a functional isoform like full-length NAB2 and quite different from NAB2ΔE3. This was further confirmed by examination of NAB2ΔE6(E82K), which showed that it did not bind Egr-1. The cellular location of NAB2ΔE6(E82K) differed from Egr-1 in the nucleus, though NAB2ΔE6 was co-localized with Egr-1, suggesting that the interaction of NAB2-ΔE6(E82K) and Egr-1 had been disrupted by the point mutation *in vivo*.

In conclusion, we found a novel regulatory system for FGF23 signaling mediated though Egr-1 and NAB2. Further study of FGF23 signaling will provide new insights into the physiological roles of FGF23 in phosphate homeostasis and skeletogenesis.

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