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An Alternative Transcript of the FOG-2 Gene Encodes a FOG-2 Isoform lacking the FOG Repression Motif

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Abstract

The FOG family of transcriptional co-factors is composed of two members in mammals: FOG-1 and FOG-2. Both have been shown to bind to GATA factors and function as transcriptional co-repressors in specific cell and promoter contexts. We have previously defined a novel repression domain localized to the N-terminus of each FOG family member, the FOG Repression Motif, which is necessary for FOG-mediated transcriptional repression. In this report, we describe the identification and characterization of a novel isoform of FOG-2 lacking the FOG Repression Motif. In contrast to full-length FOG-2, this isoform is expressed predominately in the embryonic and adult heart. It can bind GATA4 avidly, but is unable to repress GATA4-mediated activation of cardiac-restricted gene promoters. Together, these results suggest that FOG-2 repressive activity may be modulated by the generation of isoforms of FOG-2 lacking the FOG repression motif.

Keywords

Cardiac; Heart; Co-repressor; GATA4; Transcription

Introduction

Friend of GATA (FOG) proteins are multi-type zinc finger containing proteins that function as transcriptional modulators [1]. There are two FOG proteins in mammals, FOG-1 and FOG-2, and both have been shown to be required for normal mouse development. FOG-1 has been shown to play a critical role in the regulation of erythropoiesis and megakaryopoiesis [2]. Further, an endothelial-specific ablation of FOG-1 expression has demonstrated its importance in heart valve development [3]. Targeted disruption of FOG-2 in mice has revealed its role in the regulation of cardiac development, as these mice develop heart malformations that lead to the embryo's demise during mid-gestation [4,5]. FOG-2 has also been shown to be important in gonad, lung and diaphragm development [6,7].

FOG-1 and FOG-2 have an overall similar protein structure, with several highly conserved domains [1,8]. Both proteins physically interact with members of the GATA family of transcriptional activators. This interaction is mediated by multiple zinc fingers of the FOG proteins and the N-terminal zinc finger of the GATA factors [9-13]. In most cell and promoter contexts examined to date, this interaction results in the repression of GATA-mediated

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transactivation of target promoters [14-19]. There are two potential domains of FOG proteins that may mediate transcriptional repression. The first is a domain in the C-terminal portion of both FOG-1 and FOG-2 that contains a consensus binding site for C-terminal Binding Protein (CtBP), a transcriptional co-repressor family [20]. Both FOG-1 and FOG-2 have been shown to interact with CtBP-2, but the functional significance of this interaction is unclear, as disruption or deletion of this site *in vitro* or *in vivo* does not affect FOG function [9,16,21]. We have previously identified a second domain of FOG proteins localized at the N-terminus of all vertebrate FOG proteins, the FOG repression motif, which is necessary for transcriptional repression [22]. Mutations within this motif abrogate the ability of FOG-1 or FOG-2 to repress GATA-mediated transactivation of target promoters.

In this report, we describe the characterization of an alternative transcript from the FOG-2 gene that encodes a FOG-2 isoform that lacks the FOG repression motif. This protein is able to bind to GATA4, but cannot repress GATA-mediated transactivation of the atrial natriuretic factor (ANF) or B-type natriuretic peptide (BNP) gene promoters. These results suggest that alternative isoforms of FOG-2 may mediate other functions of FOG-2 aside from transcriptional repression during mouse development.

Materials and Methods

5’ Rapid Amplification of cDNA Ends (RACE) and RT-PCR

Total RNA was isolated from embryonic day 12.5 mouse hearts using Trizol (Invitrogen, Carlsbad, CA). PolyA$^+$ RNA was purified from total RNA using the NucleoTrap mRNA Purification kit (BD Biosciences, Palo Alto, CA). FOG-2S-specific nested primers (5’-TACTTGACACGGTGCAGGAAGCGACC and 5’-ACGAGCATACTTACG CATAAGAGAACC) were used in combination with a commercially available kit (CLONTECH, Palo Alto, CA) to generate 5’-RACE products. These products were cloned into pCR4-TOPO (Invitrogen) and twenty independent clones were sequenced. For tissue RT-PCR, total RNA was prepared as above from mouse embryonic day 13.5 heart, brain, liver, and hind limb as well as adult heart, brain, liver, skeletal muscle, lung, kidney, spleen, intestines, and testes. Five micrograms of each total RNA was used to prepare cDNA using Superscript II reverse transcriptase (Invitrogen). PCR was performed using this cDNA and primers specific to the FOG-2 (5’- CCTCTCATTTGCTTGCTCATCTCC and 5’-AAGTCGCCTTTCGAGATGACTTCG) and FOG-2S (5’- AAGTAATGTCGAG CTCCCACAAGG and 5’- CCGACTCTGAATCTTCCTTTCTCC) transcripts. Products were resolved using 2% agarose gel electrophoresis and visualized with ethidium bromide staining.

In Vitro Binding Assay

In vitro translated, ^{35}S-labeled FOG-2 and FOG-2S were prepared using a commercially available kit (Promega, Madison, WI) and used for *in vitro* binding assays with bacterially expressed and purified glutathione-S-transferase (GST) or GST-GATA4 fusion protein as previously described [11].

Plasmids, Cell Culture and Transfections

pcDNA3Flag/FOG-2, pcDNA-GATA4, p1.0BNPGH, p638ANFGH, and pVRβGal have been previously described [11]. pcDNA3Flag/FOG-2S was constructed using the PCR and the primers (5’-GCGAAGCTTACCATGGACTACAAAGACGATGACGACAAGAT GGACTTGAACAATAATTCCTTG and 5’-TTCAGGTGCATTTCTAGAGCTC GG) to generate a 5’ cDNA fragment encoding the FLAG epitope fused to the N-terminus of FOG-2S protein. This fragment was cloned into the Hind III/Kpn I site of pcDNA3 along with a Kpn I fragment encoding the remainder of the FOG-2S open reading frame and 3’-UTR. NIH 3T3 fibroblasts were cultured and transfected as previously described [23]. Primary cultures of

neonatal rat cardiocytes were prepared as previously described [24] and plated onto Rat Tail Collagen I coated 12-well plates (Biocoat, Fisher Scientific, Waltham, MA). Three days after plating, cardiocytes were transfected using 3 μl FuGENE 6 (Roche Applied Science, Indianapolis, IN) per well and 500ng p1.0BNPGH, 350ng pVRβGal, 0-100ng pcDNAFlagFOG-2 or pcDNAFlagFOG-2S, and pcDNA3 to a total of 1 μg plasmid DNA per well. Three days following transfection, cells and media were harvested and assayed for human growth hormone (hGH) expression, β-galactosidase activity, and protein concentration. Relative promoter activity was calculated by normalizing for transfection efficiency using β-galactosidase activity as previously described [23].

Western Analysis

NIH 3T3 fibroblasts transfected with pcDNAFlag/FOG-2 or pcDNAFlag/FOG-2S were harvested 3 days after transfection and 75 μg of total cell lysates resolved by 6% SDS-PAGE followed by western transfer to a nitrocellulose membrane. This membrane was blocked with Blotto (10 mM Tris, pH7.5, 140 mM NaCl, 0.05% Tween-20, 5% powdered milk) for 1 hour at room temperature, followed by incubation with 1:1000 dilution of an anti-FOG-2 rabbit polyclonal antibody (M247, Santa Cruz Biotechnology, Santa Cruz, CA) in Blotto. The membrane was washed with TBST (10 mM Tris, pH7.5, 140 mM NaCl, 0.05% Tween-20) incubated for 1 hour with a 1:5000 dilution of goat anti-rabbit antibody conjugated to horseradish peroxidase. The blot was washed extensively and developed using a commercially available kit (ECL-plus, GE Healthcare, Piscataway, NJ).

Results and Discussion

Isolation of an alternative transcript of murine FOG-2

In the course of screening a mouse embryonic heart cDNA library for full-length clones of FOG-2, we obtained 2 identical cDNA clones containing alternative sequences at the 5' end of the FOG-2 cDNA. These clones were identical to the previously reported FOG-2 cDNA (GenBank accession number **NM 011766**) from base pairs (bp) 544 to 4992, but had 240 bp of distinct 5' sequence. To identify the 5' end of this cDNA, we used 5' RACE with primers to this unique sequence (Figure 1). Subsequently, RACE products were cloned and 20 independent clones were sequenced. The longest of these clones revealed an extension of an additional 1125 bp. When assembled, this message, here designated FOG-2S (GenBank accession number **EF514219**), is 5823 bp in length and has an open reading frame with a start methionine at 1722 bp, encoding a protein of 1019 amino acids. This protein is identical to FOG-2 except it lacks the N-terminal 132 amino acids where the FOG repression motif is located (Figure 1). It contains all of the zinc finger motifs, the C-terminal binding protein (CtBP) interaction domain, and the nuclear localization domain previously characterized in FOG-2 [16].

Given that the FOG-2 and FOG-2S transcripts have distinct 5' sequences and identical 3' sequence, it suggests that these transcripts are transcribed from the same gene from alternative transcriptional start sites. Indeed, alignment of the distinct 5' sequence of FOG-2S to the mouse genome identifies a novel exon located between exon 1 and 2 of the FOG-2 gene (data not shown). This result confirms that the FOG-2S transcript is indeed being generated from the FOG-2 gene and suggests the existence of an alternative promoter within the first intron of this gene that drives expression of the FOG-2S transcript.

To determine the pattern of expression of FOG-2S, we took an RT-PCR approach. We designed 5' primers specific to FOG-2 and FOG-2S as well as 3' primers designed to a region common to both messages. RT-PCR was then performed using these primers and RNA generated from multiple tissues (Figure 2). The results show that FOG-2 is expressed in multiple tissues

including heart, brain, lung, spleen and testes, consistent with previously reported northern analysis [11]. In contrast, FOG-2S was predominately detected in embryonic and adult heart, with very faint levels in embryonic and adult brain. This result demonstrates that the FOG-2S transcript is more restricted in its pattern of expression when compared to the FOG-2 transcript and is consistent with the notion that these two transcripts are generated from differentially regulated promoters within the FOG-2 gene. Northern analysis with 5 μg of adult heart polyA$^+$ RNA and a probe specific to FOG-2S failed to detect a message, suggesting that this transcript is expressed at very low levels in the adult heart.

FOG-2S physically interacts with GATA4

To investigate the function of the protein isoform encoded by FOG-2S, we first sought to demonstrate that this protein could physically interact with GATA4. We have previously shown that multiple zinc fingers domains of FOG-2 are able to physically associate with the N-terminal zinc finger of GATA4 [16]. Since the zinc finger domains of FOG-2 and FOG-2S are identical, we would predict that FOG-2S would be able to bind GATA4. To demonstrate that FOG-2S can bind to GATA4, we used an *in vitro* binding assay (Figure 3). Bacterially expressed glutathione S-transferase (GST) or GST fused to the zinc finger domains of GATA4 (GST-GATA4) were purified and incubated with *in vitro* translated, ^{35}S-labeled FOG-2 or FOG-2S. Resultant complexes were washed extensively and resolved by SDS-PAGE. As expected, both FOG-2 and FOG-2S bind to GST-GATA4, but not GST alone, confirming that FOG-2S can physically interact with GATA4.

FOG-2S does not repress GATA4 transactivation

We have previously shown that the FOG proteins mediate transcriptional repression via a domain localized at their N-terminus, the FOG repression motif. Since FOG-2S lacks the FOG repression motif (see Figure 1), we predicted that it would be unable to repress GATA-mediated transactivation of cardiac promoters. To test the functional significance of the FOG-2S/GATA4 interaction, we transiently transfected murine NIH 3T3 fibroblasts with a reporter construct containing 638 bp of the rat ANF promoter driving human growth hormone expression. In addition, expression plasmids encoding GATA4, FOG-2, or FOG-2S were added and cells were assayed for growth hormone expression 48 hours post-transfection. As can be seen in Figure 4A, GATA4 transactivated the ANF promoter approximately 200-fold over background and FOG-2 repressed this transactivation up to 73-fold in a dose dependent fashion (compare columns 2-5). In contrast, the addition of FOG-2S did not significantly change the activity of the ANF promoter in the presence of GATA4 (compare columns 2 and 6-8), suggesting that FOG-2S cannot function as a transcriptional co-repressor. Western analysis of cell lysate transfected with each expression construct demonstrated similar levels of FOG-2 and FOG-2S expression (Figure 4B).

It is possible that fibroblasts lack some necessary co-factor for FOG-2S function. Since FOG-2S is expressed in the heart, we isolated primary neonatal cardiomyocytes and transduced them with a reporter plasmid containing the B-type natriuretic peptide (BNP) promoter driving expression of human growth hormone. In addition, we also transduced these cells with expression plasmids for FOG-2 or FOG-2S. As can be seen in figure 4C, the BNP promoter is active in these cells and the addition of FOG-2 repressed the activity of the BNP promoter up to 56% in a dose dependent fashion (compare columns 1-4). In contrast, the addition of FOG-2S did not alter the activity of the BNP promoter, demonstrating that FOG-2S is unable to mediate transcriptional repression in this cell and promoter context. Taken together, these results suggest that FOG-2S physically interacts with GATA4, but fails to repress GATA4-mediated transactivation of target promoters.

The function of FOG-2S during development remains unclear. Given its more restricted pattern of expression, it is tempting to speculate that this isoform may play a role in cardiac development. Previously reported targeted disruptions of the FOG-2 gene affected both the FOG-2 and FOG-2S transcripts, so it is not clear what role FOG-2S might be playing during cardiac development. Further work will be necessary to more clearly distinguish the role of FOG-2S during cardiac morphogenesis.

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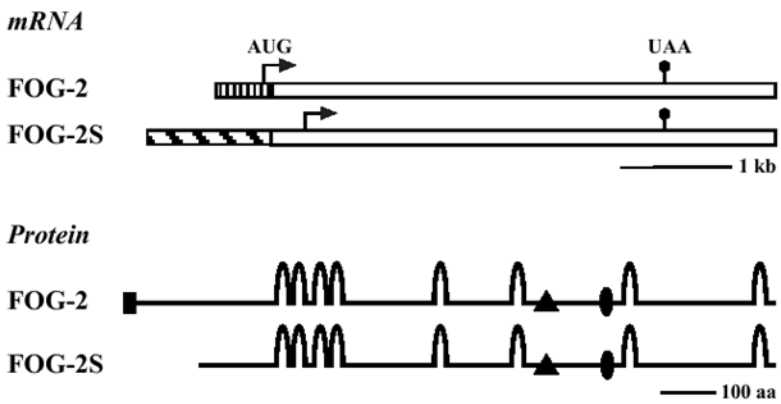

Figure 1. Alternative Transcripts of the Murine FOG-2 Gene

Shown above is a schematic of the FOG-2 and FOG-2S transcripts. The distinct 5'ends of each transcript are shaded. The translational start site for each transcript is indicated by the arrow and "AUG", while the translational stop site is indicated by a shaded octagon and "UAA". Below is a schematic of the predicted primary structure of the FOG-2 and FOG-2S protein isoforms. Zinc finger motifs are indicated by open loops. The nuclear localization signal is indicated by the closed triangle, while the closed oval indicates the CtBP-interaction motif. The FOG repression motif is indicated by the closed square.

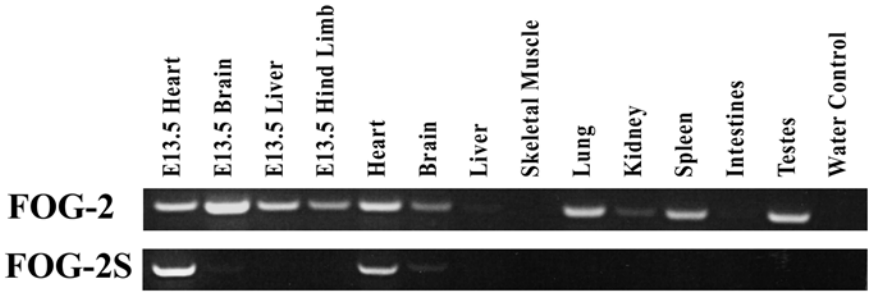

Figure 2. Expression of the FOG-2 and FOG-2S Transcripts

RT-PCR was performed using primers specific to the FOG-2 (top panel) or FOG-2S (bottom panel) transcripts and cDNA from mouse embryonic day 13.5 (E13.5) and adult tissues as indicated above. Products were resolved using 2% agarose gel electrophoresis and visualized using ethidium bromide staining.

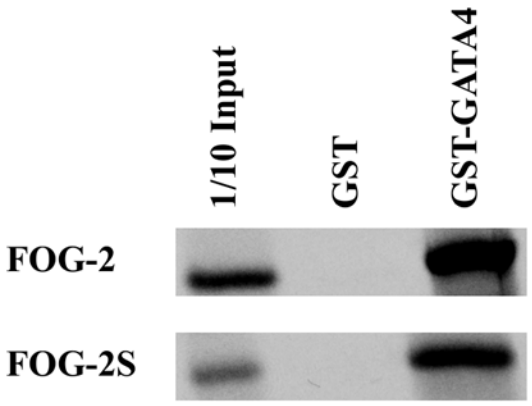

Figure 3. FOG-2S Physically Associates with GATA4

In vitro binding assays were performed using purified glutathione-S-transferase (GST) or GST-GATA4 fusion protein with ^{35}S-labeled, *in vitro* translated FOG-2 (top panel) or FOG-2S (bottom panel). Complexes were isolated using glutathione-sepharose beads, resolved using SDS-PAGE, and detected by autoradiography. As a control, 10% of input of each FOG-2 isoform is shown on the left.

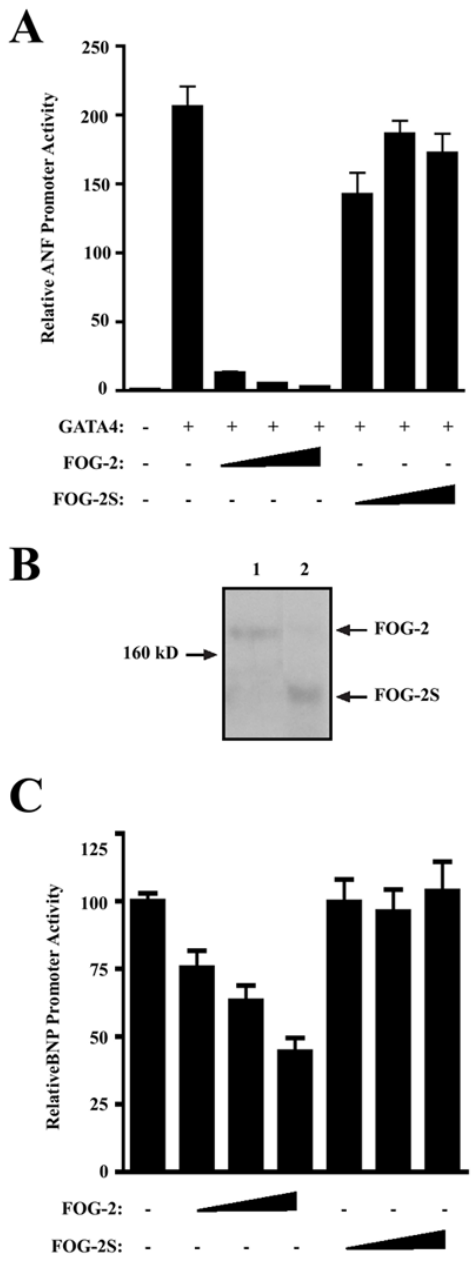

Figure 4. FOG-2S fails to repress Transcription of GATA4-dependent Promoters

In A, NIH 3T3 fibroblasts were transfected with a reporter plasmid containing 638bp of the ANF promoter driving expression of human growth hormone (hGH) and expression plasmids for GATA4, FOG-2, and FOG-2S as indicated. Forty-eight hours after transfection, cells were harvested and assayed for hGH expression. Results were normalized for transfection efficiency and are reported as the mean ± S.E.M. (n=4). In B, western analysis was performed on cell lysates of 3T3 fibroblasts transfected with expression vectors for FOG-2 (lane 1) or FOG-2S (lane 2) using an anti-FOG-2 antibody. In C, primary neonatal cardiomyocytes were transfected with a reporter plasmid containing 1 kb of the BNP promoter driving expression of hGH along with expression vectors for FOG-2 or FOG-2S as indicated. Forty-eight hours after

transfection, cells were harvested and assayed for hGH expression. Results were normalized for transfection efficiency and are reported as the mean ± S.E.M. (n=8).