hFOG-2, a Novel Zinc Finger Protein, Binds the Co-repressor mCtBP2 and Modulates GATA-mediated Activation*

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We have identified a novel human zinc finger protein, hFOG-2, which is related to but distinct from the murine transcription factor Friend-of-GATA-1 (mFOG-1). The hFOG-2 gene was initially detected in K562 cells using a polymerase chain reaction approach with degenerate primers corresponding to zinc finger regions of mFOG-1. A murine homologue of hFOG-2 was also identified in the mouse expressed sequence tag data banks, indicating that a family of FOG genes exists in mammals. hFOG-2 appears to be widely expressed, while mFOG-1 is expressed primarily in erythroid and megakaryocytic cells and plays a fundamental role in the development of these lineages. Sequencing of the full-length hFOG-2 cDNA indicates that the interaction domains for transcription factors GATA-1 and mCtBP2 are both conserved and we have shown that this new FOG protein also physically interacts with these factors. We have demonstrated that hFOG-2, like mFOG-1, can act in concert with GATA-1 to activate gene expression from the p45 NF-E2 promoter region, but that it can also act to repress GATA-mediated activation of additional reporter constructs. Finally, we have identified a repression domain in hFOG-2 and shown that repression is dependent upon the integrity of the mCtBP2 interaction motif Pro-Ile-Asp-Leu-Ser.

GATA-1 is the founding member of the GATA family of transcription factors that bind to (T/A)GATA(A/G) motifs in DNA via C4-type zinc fingers. GATA motifs are found within the regulatory regions of many genes, and it has been suggested that GATA-1 is involved in the regulated expression of most, if not all, erythroid-specific genes (reviewed in Ref. 1 and 2). Gene inactivation experiments have shown that GATA-1 is essential for red blood cell formation (3, 4) and consequently for mouse embryo survival (5). GATA-1 contains two zinc fingers and an N-terminal transcriptional activation domain (6). The C-terminal finger is essential for DNA binding, while the N-terminal finger stabilizes this interaction (7).

Several findings hinted at the possibility that GATA-1 recruits a co-factor in hematopoietic cells. First, a truncated GATA-1, lacking the trans-activation domain, is still capable of megakaryocytic conversion of the myeloid precursor cell line, 416B (8), and rescue of terminal maturation of GATA-1−/− erythroid precursors (9). In addition, although the N-terminal zinc finger is not essential for DNA binding, it is absolutely required for terminal erythroid maturation (9). In an effort to identify co-factor proteins that interacted with the N-terminal zinc finger of GATA-1, a yeast two hybrid screen was performed and resulted in the isolation of the transcriptional cofactor Friend-of-GATA (mFOG-1) (10). mFOG-1 is highly expressed in erythroid and megakaryocytic cell lines and in the spleen, liver, and testis (10). Recent work has shown that mFOG-1 plays a critical role during hematopoiesis. The targeted disruption of mFOG-1 in mice leads to embryonic lethality, with mice dying of severe anemia between embryonic day 11 and 12 (11). Examination of the mFOG-1−/− embryos revealed that overall development was not grossly affected, indicating instead a specific failure of primitive hematopoiesis. mFOG-1−/− embryos were impaired in both primitive and definitive erythropoiesis, and no megakaryocytes or megakaryocytic markers could be detected indicating an extremely early block in megakaryocytic development.

Several findings attest to mFOG-1 being a genuine, in vivo co-factor of GATA-1. Apart from showing that these factors specifically interact and are expressed in parallel during embryonic development, they were also shown to co-operate in cell lines to promote erythroid and megakaryocytic differentiation (10). Differences in the defects seen in knockout mice, however, suggest that mFOG2 may also play a role in megakaryocyte lineage commitment that is independent of GATA-1 (11).

mFOG-1 has nine zinc fingers with four of the TFIIfA-like CCHH-type and five of the variant CCHC-type. Zinc fingers may be involved in protein:DNA or protein:protein interactions. Although mFOG possesses numerous zinc fingers of a type that has been shown to interact with DNA (12), mFOG has not yet been shown to bind DNA directly. In the absence of a specific DNA-binding domain, mFOG might be recruited to a consensus GATA DNA target via its interaction with GATA-1. Indeed, transfection experiments in mammalian cells have demonstrated the synergistic activation of transcription from the hematopoietic-specific p45 NF-E2 regulatory region by mFOG-1 and GATA-1 (10).

As well as functioning as a co-activator, there is evidence that mFOG-1 may be involved in negatively regulating gene expression. Recently, mFOG-1 was shown to interact with the transcriptional co-repressor mCtBP2 (13). The founding mem-

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‡ The abbreviations used are: GOG, Friend-of-GATA; FBS, fetal bovine serum; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; kb, kilobase pair(s); GST, glutathione S-transferase; GH, growth hormone.
umber of the CtBP family of proteins, hCtBP1, was initially identified as a protein which bound to the C-terminal region of the adenovirus E1a protein, modulating its function (14). The list of mammalian proteins that specifically associate with CtBP2 (highly related to hCtBP1) has now been extended to include mCtBP1, BKL, AEB6, ZEB, and Evi-1 (13). mCtBP2 binds to a region of mFOG-1 containing the CtBP interaction motif Pro-Leu-Asp-Leu-Ser (N-terminal to zinc finger 7), which is distinct from the zinc finger to which GATA-1 binds (zinc finger 6; Ref. 15). Thus, the possibility exists that mFOG-1, on binding to mCtBP2, becomes associated with a repressor complex.

The Drosophila factor U-shaped (16) has limited homology to mFOG-1 in its zinc finger regions. U-shaped also interacts with a GATA factor, Panner, and this interaction has been shown to play an important role in Drosophila bristle cell formation and proneural gene expression (16, 17). It has been proposed that mFOG-1 and U-shaped belong to a class of factors that modulate the transcriptional activity of GATA proteins. Given that different GATA factors (i.e., GATA-2 to -6) are widely expressed in mammalian tissues, and particularly in tissues in which mFOG-1 is not expressed, we were interested in determining whether additional FOG-like genes existed in mammals. Here we report the identification, cloning, and characterization of the second member of the mammalian FOG family, hFOG-2.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Growth Conditions**—The human cell lines Meg-01, CHRF-288, and Dami/HEL were cultured in Iscove’s modified Dulbecco’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS), streptomycin, and penicillin. Similarly, the cell lines Mo7e-IL3 and Mo7e-TPO were also cultured in Iscove’s modified Dulbecco’s medium except that their medium contained 15% FBS and was supplemented with 20 ng/ml interleukin-3 and 20 ng/ml thrombopoietin, respectively. The majority of the other human cell lines were cultured in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FBS, streptomycin (100 

**In Vitro Binding Assays**—Full-length hFOG-2 was cloned into pcDNA3 (Invitrogen), and 35S-labeled hFOG-2 was generated by in vitro transcription and translation using T7 polymerase and the TNT system (Promega). 35S-Labeled mCtBP2 and GATA-1 were generated as described previously (13, 23). GST fusion proteins and binding assays were carried out as described previously (24).

**RESULTS**

**Isolation of hFOG-2, a Second Member of the FOG Family—** Homology between mFOG-1 and Drosophila U-shaped is essentially restricted to the zinc finger motifs of the CCHC-type. When the zinc fingers of this type are aligned, approximately 50% of the amino acids are identical (up to 68% identity when mFOG-1 zinc finger 6 and the fourth U-shaped zinc finger are compared). In contrast, zinc fingers of the CCHH-type typically show 25% identity. Therefore, we designed degenerate primers corresponding to several zinc fingers of the CCHH-type, mFOG-1 fingers 5, 6, 7, and 9, as we reasoned that these regions might be conserved in other FOG-like genes. Initially, the optimal PCR conditions for each primer combination was determined using cDNA from the murine cell line MEL, which was known to express mFOG-1. We repeated these PCR reactions using K562 cDNA as the starting material. Primers F2 and R1 resulted in the amplification of a product of approxi-
mately 0.5 kb, similar to that expected for a human equivalent of mFOG-1. This cDNA fragment, designated F2-R1 (see underlined region in Fig. 1A), was cloned and sequenced, and data bank searches revealed that it overlapped two other sequences, one in each of the human and murine EST data banks (GenBank accession nos. AA442019 and AA231039, respectively). The putative proteins encoded by these partial cDNAs show greater than 93% identity. They also show significant homology to mFOG-1 zinc fingers 5, 6, and 7. The existence in mice of both mFOG-1 and the cDNA AA231039 indicates that the

![Fig. 1. The hFOG-2 protein sequence.](image)

A. The deduced amino acid sequence of the cDNA clone, GenBank AF119334. Putative zinc fingers of both CCHH and CCHC type motifs are boxed. The original fragment, identified by PCR using the degenerate primers F2 and R1, is underlined. The positions of the 5'-RACE and 3'-RACE primers, used to generate the full-length cDNA, are indicated. The putative CtBP interaction motif PIDLS is shown in gray.

B. An alignment of hFOG-2 and mFOG-1 protein sequences. Identical amino acids are indicated with asterisks. The putative zinc fingers are underlined in mFOG-1 and overlined in hFOG-2. The numbers correspond to GenBank accession numbers AF119334 (hFOG-2) and AF006492 (mFOG-1).
differences between our F2-R1 clone and mFOG-1 were not purely due to sequence divergence between equivalent genes in mice and humans. Instead, we propose that clone F2-R1 is a partial cDNA corresponding to a second mammalian FOG family member. Accordingly, we refer to the original FOG gene as mFOG-1 and our new gene as hFOG-2. 5

Partial cDNA corresponding to a second mammalian FOG family member was previously demonstrated that mFOG-1 functions with GATA-1 to co-activate the p45 NF-E2 promoter (10). A similar activity is also observed with hFOG-2 (Fig. 2). GATA-1 alone has little effect when co-transfected with a reporter harboring 7 kb of the p45 NF-E2 promoter upstream of growth hormone (GH). However, co-expression of both GATA-1 and hFOG-2 (from pcDNA3) activates this promoter approximately 3-fold.

Interaction of hFOG-2 with CtBP—Although both mFOG-1 and hFOG-2 have been shown to act as co-activators for mGATA-1 (10), the Drosophila FOG-like protein, U-shaped, is well characterized as negative regulator of the Drosophila GATA factor Pannier (17). It is possible that the mammalian FOG family members may also repress transcription in certain contexts, perhaps through interaction with co-repressors of the CtBP-family, mCtBP2, a co-repressor for various hematopoietic transcription factors (13), has been shown to interact with a region of mFOG-1, N-terminal to zinc finger 7, which contains the CtBP interaction motif Pro-Ile-Asp-Leu-Ser (13). Interestingly, this motif is conserved in hFOG-2 in the same position relative to the seventh zinc finger, within a region that otherwise shares little similarity to mFOG-1 (see Fig. 1B). This suggests that CtBP proteins may also interact with hFOG-2. Using both in vitro pull-down assays and the yeast two hybrid system, we have tested the interaction of mCtBP2 and a hFOG-2 subfragment similar to the mFOG-1 fragment shown to interact with mCtBP2. This fragment (FOG-2CBD), spanning amino acids 809–852 of hFOG-2, contains the putative CtBP-binding domain.

When FOG-2CBD is fused with GST and immobilized on agarose beads, it is able to efficiently retain 35S-labeled mCtBP2, while GST protein alone does not bind mCtBP2 (Fig. 5A, lanes 3 and 2, respectively). It has previously been shown that mutation of the CtBP interaction motif within various transcription factors, from Pro-X-Asp-Leu-Ser to Pro-X-Ala-Ser, abolishes their interaction with CtBP proteins (13, 27). The corresponding mutation in FOG-2CBD, creating FOG-2CBD(MUT), completely abolishes interaction with mCtBP2 in this in vitro assay (Fig. 5A, lane 4), demonstrating that the integrity of the Pro-Ile-Asp-Leu-Ser motif is required for the interaction of mCtBP2 with FOG-2CBD.

To test whether full-length hFOG-2 could also interact with mCtBP2, we generated a GST fusion protein with mCtBP2 and assessed its ability to retain full-length 35S-labeled hFOG-2 in an in vitro pull-down assay. As shown in Fig. 5B, GST alone does not retain full-length hFOG-2 (lane 2), whereas a strong interaction is observed with a GST-mCtBP2 fusion (lane 3).
Hence, full-length hFOG-2 is able to interact with mCtBP2. We next determined whether this interaction could also be observed in the yeast two-hybrid assay. Yeast expressing both the gal4AD-FOG-2CBD and gal4DBD-mCtBP2 hybrids grow on media lacking His, Leu, and Trp (SD-H-L-T), indicating interaction between mCtBP2 and this fragment of hFOG-2 (Fig. 5C). The requirement of the Pro-Ile-Asp-Leu-Ser motif for hFOG-2 interaction with mCtBP2 is again highlighted by the inability of the yeast transformants harboring gal4DBD-mCtBP2 and gal4-FOG-2CBD(MUT) to grow on the SD-H-L-T media. We also tested the interaction of FOG-2CBD with another member of the CtBP family, hCtBP1. Again, in the yeast system we observed a strong interaction between these two factors, dependent on the integrity of the Pro-Ile-Asp-Leu-Ser motif in FOG-2CBD (data not shown). Hence, FOG2CBD is able to interact with both hCtBP1 and mCtBP2.

hFOG-2 Repression via the Co-repressor CtBP—To test whether the hFOG-2 subfragment harboring the CtBP binding domain can mediate repression, we utilized a gal4DBD-FOG-2CBD fusion protein such that FOG-2CBD is directed to a gal4-dependent promoter driving GH gene expression. We tested the ability of this fusion protein to repress expression in the cell line NIH-3T3, which harbors endogenous CtBP proteins. Fig. 6A shows the results of these experiments. The fusion protein efficiently represses GH expression in a dose-dependent manner (columns 5–7), whereas the gal4DBD has little effect on promoter activity (columns 2–4). Repression by gal4DBD-FOG-2CBD(MUT) is significantly impaired (columns 8–10), suggesting that interaction with the co-repressor CtBP is necessary for this dose-dependent repression. Therefore, we have mapped a small repression domain in hFOG-2 to a region overlapping the Pro-Ile-Asp-Leu-Ser motif, between amino acids 809 and 852, and have shown that repression is dependent on the integrity of this motif.

To verify this repression in the context of full-length hFOG-2, we developed an assay to test hFOG-2’s effect on GATA-1-dependent activation of the M1a promoter, containing one GATA-1 site from the murine α-globin promoter, upstream of a minimal TATA box and the GH gene. GATA-1 activates one GATA-1 site from the murine α-globin promoter, upstream of a minimal TATA box and the GH gene. GATA-1 activates this promoter (Fig. 6B, column 2). However, when GATA-1 is co-expressed with increasing amounts of full-length hFOG-2, GATA-1 activation is dose-dependently repressed to approximately 3-fold (Fig. 6B, columns 7–10). This effect is also seen with mFOG-1 (columns 3–6).

The residues within the N-finger of GATA-1 responsible for interaction with mFOG-1 are conserved among all GATA fac-
Thus, we reasoned that hFOG-2 may also play a role in repressing the activity of other GATA family members. To test this, we co-expressed GATAs -1, -2, and -4 with hFOG-2 and compared its ability to repress GATA-mediated activation (Fig. 6C). hFOG-2 efficiently represses all three GATA factors, and this result suggests hFOG-2 may function to modulate activities of GATA family members in tissues where FOG-1 expression is absent.

**DISCUSSION**

**FOG-2 Is Related to but Distinct from FOG-1**—In this study, we sought to determine whether a family of FOG transcription factors exists in humans. We have identified a novel factor, hFOG-2, and have shown that it is related to mFOG-1, not just at the amino acid level but in its ability to associate with certain protein partners. We have shown that like mFOG-1, hFOG-2 physically interacts with GATA-1 (the N-finger but not the C-finger of mGATA-1) and the co-repressor CtBP (mCtBP2 and hCtBP1). Interestingly, the *Drosophila* FOG-like protein U-shaped interacts with the GATA factor Pannier, and the presence of a putative CtBP interaction domain within U-shaped suggests that it too may interact with this factor (13). However, unlike U-shaped, which displays little homology to the FOG-like factors outside its zinc finger regions, an alignment of hFOG-2 and mFOG-1 clearly demonstrates the conservation of amino acid sequence (40% identity overall) across their entire lengths and hints at a common ancestry. The structural requirements of certain regions to enable interaction with shared interaction partners will of course put constraints on sequence divergence. Examples of this may be the highly conserved sixth zinc fingers of hFOG-2 and mFOG-1, which interact with GATA-1, and the Pro-Ile-Asp-Leu-Ser motifs responsible for interaction with CtBP. It is interesting that high level homology extends to several of the other CCHC-type fingers, and we are currently testing the hypothesis that they too may be involved in interacting with GATA proteins.

Several regions within the FOG proteins are notable for their
differences. The double zinc finger, mFOG-1 fingers 2 and 3, which are separated by the evolutionarily conserved H/C-link motif found in many Krüppel-like factors (28), is absent in hFOG-2. In addition, the region between the seventh and last zinc fingers of the two FOG factors differ; hFOG-2 has an additional 138 amino acids separating these fingers, yet a finger corresponding to mFOG-1 zinc finger 8 is absent in hFOG-2. This region shows no significant homology to known proteins. Finally, the proline-rich regions described for mFOG-1 are not generally conserved in hFOG-2. As a consequence, hFOG-2 and mFOG-1 may differ with respect to their interaction with protein partners or possibly, their DNA targets.

**The Mechanism of Action of FOG Proteins**—Neither FOG factor has yet been shown to interact with DNA directly, and it has been proposed that FOG is recruited to the regulatory regions of certain genes primarily through its association with GATA factors (10, 11). The FOG interaction and DNA interaction faces of GATA-1 are found on opposite sides of the N-terminal zinc finger and it is therefore assumed that FOG will piggy-back onto GATA-1 and not compromise its DNA binding activity (15). However, FOG may alter GATA binding through a change in protein conformation or through a direct DNA association such that FOG-GATA complexes may bind only a subset of GATA consensus sites. It is also possible that FOG and GATA are involved in long range associations, bringing enhancer and promoter regions together via their interactions.

Previously, transfection studies in mammalian cells showed that mFOG-1 and GATA-1 can synergistically transactivate expression from the regulatory region of the hematopoietic-specific gene, p45 NF-E2 (10). Likewise, we have shown that hFOG-2 can also act in a similar manner. In contrast, both hFOG-2 and mFOG-1 reduced the ability of GATA-1 to activate transcription of the M1α promoter, a reporter containing the mouse α-globin GATA site. We have mapped a small repression domain in hFOG-2 to a region encompassing the Pro-Ile-Asp-Leu-Ser motif (necessary for interaction with CtBP), and have shown that repression is dependent on the integrity of this motif. This suggests that upon interaction of hFOG-2 with the CtBP co-repressors, hFOG-2 may be involved in down-regulating expression of GATA-target genes. Prior to this, mFOG-1 was believed to function solely as a co-activator. We have now shown that FOG proteins can also act to repress transcription, at least in part through the recruitment of the co-repressor CtBP. It is likely that mFOG-1 and hFOG-2 may act as molec-
Co-expression of FOG and GATA factors may not always reflect their functional interaction. For example, although mFOG-1 is co-expressed with mGATA-2 and mGATA-3 in multipotential progenitor and T-lymphocyte cell lines, respectively, and it has been shown to physically interact with these factors in the yeast two-hybrid system, it is believed that mFOG-1 is not an essential functional co-factor for these GATA factors (10, 11). This conclusion follows from the observation that multipotential progenitors, mast cells, and T-lymphocytes that are known to rely on mGATA-2 and mGATA-3 in vivo are not affected in the mFOG-1 knockout (11, 18, 19, 29). It remains possible, therefore, that there is limited redundancy in the few tissues in which the two proteins are co-expressed. Further genetic studies in mice will help illuminate the specific functions of different members of this family.

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FIG. 6. hFOG-2 represses GATA-mediated activation, and contains a minimal repression domain which utilizes the co-repressor CBP. A, a gal4DBD-FOG2CBD fusion, which contains the CBP interaction motif, represses transcription, while mutation of this motif retains a minimal repression domain which utilizes the co-repressor CtBP. 

![Diagram](image-url)

B Cloning and Characterization of hFOG-2

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