A Functionally Conserved N-terminal Domain of the Friend of GATA-2 (FOG-2) Protein Represses GATA4-Dependent Transcription*

Received for publication, February 23, 2000, and in revised form, April 11, 2000 Published, JBC Papers in Press, May 2, 2000, DOI 10.1074/jbc.M001522200

Eric C. Svensson‡, Gordon S. Huggins§, Fred B. Dardik‡, Christine E. Polk‡, and Jeffrey M. Leiden‡

From the ‡Department of Medicine, University of Chicago, Chicago, Illinois 60637 and §The Laboratory of Cardiovascular Biology, Harvard School of Public Health and Harvard Medical School, Boston, Massachusetts 02115

GATA4 is a transcriptional activator of cardiac-restricted promoters and is required for normal cardiac morphogenesis. Friend of GATA-2 (FOG-2) is a multizinc finger protein that associates with GATA4 and represses GATA4-dependent transcription. To better understand the transcriptional repressor activity of FOG-2 we performed a functional analysis of the FOG-2 protein. The results demonstrated that 1) zinc fingers 1 and 6 of FOG-2 are each capable of interacting with evolutionarily conserved motifs within the N-terminal zinc finger of mammalian GATA proteins, 2) a nuclear localization signal (RKKRRK) (amino acids 736–740) is required to program nuclear targeting of FOG-2, and 3) FOG-2 can interact with the transcriptional co-repressor, C-terminal-binding protein-2 via a conserved sequence motif in FOG-2 (PIDLS). Surprisingly, however, this interaction with C-terminal-binding protein-2 is not required for FOG-2-mediated repression of GATA4-dependent transcription. Instead, we have identified a novel N-terminal domain of FOG-2 (amino acids 1–247) that is both necessary and sufficient to repress GATA4-dependent transcription. This N-terminal repressor domain is functionally conserved in the related protein, Friend of GATA1. Taken together, these results define a set of evolutionarily conserved mechanisms by which FOG proteins repress GATA-dependent transcription and thereby form the foundation for genetic studies designed to elucidate the role of FOG-2 in cardiac development.

The GATA family of transcriptional activators contains six mammalian members (GATA1–6) (1–7). Four of the mammalian GATA proteins are known to play critical roles in the development of the hematopoietic (GATA1–3) (8–10) and cardiovascular systems (GATA4) (1, 11–14). GATA4 is expressed at high levels in both embryonic and adult cardiomyocytes and can bind to and activate the expression of multiple cardiac-restricted promoters and can include those from the cTnC, ANF, MHC, and myosin heavy chain (MHC) genes (5, 15–17). GATA4-deficient mice produced by gene targeting die at embryonic day 8.5 with severe defects in early cardiac morphogenesis (18, 19). All of the mammalian GATA proteins contain a DNA binding domain composed of two evolutionarily conserved zinc fingers (N- and C-terminal) and bind to the consensus DNA sequence WGATAR and its variants (20, 21). Previous studies have demonstrated that several transcription factors including erythroid Kruppel-like factor, specificity protein 1, Nkx2.5, and nuclear factor of activated T cells 3 can associate specifically with the C-terminal zinc fingers of the GATA proteins resulting in synergistic transcriptional activation (3, 22–25). The N-terminal zinc fingers of GATA proteins have also been shown to mediate functionally important protein-protein interactions. The structurally related zinc finger proteins U-shaped and Friend of GATA1 (FOG)1 were both identified by their ability to bind to the N-terminal zinc finger of the GATA proteins Pannier and GATA1, respectively. Genetic studies demonstrated that U-shaped represses the transcriptional activity of the Drosophila GATA protein (26). FOG can function as either a transcriptional co-activator or repressor of GATA1 depending on the cell and promoter context being assayed (27–31).

We and others have recently identified a novel FOG family member, Friend of GATA-2 (FOG-2), that is structurally related to both FOG and U-shaped (29, 32–34). FOG-2 is an 1151-amino acid polypeptide that contains 8 zinc finger motifs and is expressed in the heart, brain, and testis in adult mice. During mouse embryonic development, FOG-2 is first expressed in the developing heart and septum transversum at embryonic day 8.5. It is subsequently expressed in the urogenital ridge, developing gut, and brain (33, 34). In each of these tissues its pattern of expression overlapped with that of one or more GATA proteins. FOG-2 specifically associates with the N-terminal zinc finger of GATA4 and represses GATA4-mediated transcriptional activation of several cardiac-restricted promoters including those from the ANF, BNP, and cTnC genes (33, 34).

In the studies described in this report, we have used in vitro mutagenesis to functionally characterize the FOG-2 protein and to better understand the mechanisms by which it represses GATA-dependent transcription from cardiac promoters. Our results demonstrate that multiple zinc fingers of FOG-2 are capable of interacting with the N-terminal zinc finger of GATA4. Single amino acid substitutions in conserved residues of the N-terminal zinc finger of GATA4 can abolish FOG-2 binding and transcriptional repression without disrupting the ability of GATA4 to bind to DNA or to activate transcription. We have demonstrated that FOG-2 can physically associate with a potent transcriptional co-repressor, C-terminal-binding...

* This work was supported in part by Grant HL54592 from the NHLBI, National Institutes of Health (to J. M. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Harvard School of Public Health, Bldg. II, Rm. 117, 677 Huntington Ave., Boston, MA 02115. Tel.: 617-432-3444; Fax: 617-432-3794; E-mail: leiden@cvlab.harvard.edu.

1 The abbreviations used are: FOG, Friend of GATA1; CtBP2, C-terminal-binding protein-2; aa, amino acids; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase(s); GST, glutathione S-transferase; cTnC, cardiac troponin C; ANF, atrial natriuretic factor; BNP, brain natriuretic peptide.
protein-2 (CtBP-2). However, this association is not required for FOG-2 to repress the GATA-mediated transcriptional activation of cardiac-restricted promoters. Instead, we have identified a non-CtBP-2-dependent repression domain of FOG-2 located at the N terminus of the protein (aa 1 to 247). This domain is both necessary and sufficient for FOG-2-mediated repression of GATA4-dependent transcription and is functionally conserved in the N-terminal region of FOG. Furthermore, when fused to a Gal4 DNA binding domain, the N-terminal repressor domain of FOG-2 can repress transcription from an SV40 promoter juxtaposed to Gal4 DNA binding sites. Taken together, these results help to elucidate the molecular mechanisms by which FOG-2 functions to repress GATA4-dependent cardiac transcription and provide the structural basis for ongoing studies designed to understand the biochemical basis of FOG-2-mediated regulation of cardiomycyte gene expression.

MATERIALS AND METHODS

Plasmids—pCDNA3-GATA4, pGEX-GATA4, and pCDNA3-FOG-2 have been described previously (33). pSV40Gal4Luciferase containing four copies of the Gal4 upstream activating sequence 5'- of the SV40 promoter was the generous gift of Dr. D. A. Capistrano (University of Pennsylvania). pCDNA3-Nx2.5 was constructed by using the PCR and primers (5'-GAGAATTCTGATTGGGTGAC-GCAGAATCTGC and 5'-CTCGAGGAACAGGACGGACCTTTG) to generate a 521-bp fragment of the 5'-coding region of Nx2.5, which was then cloned into the EcoRI/XhoI site of pCDNA3. A 1.9-kb XhoI fragment from pBS-Csx was then inserted into the XhoI site of this plasmid. Point mutations in the N-terminal zinc finger of GATA4 were created using PCR-directed mutagenesis with the following oligonucleotides: 5'-GCGTCGGAGAAGCAGTGTCCTCTCCACTCG and 5'-GCTCTAGACGACAGAAGCAGAAGCAGCATC to generate a 257-bp fragment that was cloned into the BamHI/XhoI sites of pCDNAFlag. Subsequently, a 3.5-kb XhoI fragment from the FOG-2 cDNA (1208–4770 bp) was cloned into the XhoI site of FOG-2 cDNA (534–1151) primers (5'-GTTGATCCTCCTCCCTCTCCGTGAGAAGC and 5'-TCTTCCACGACGATCCTCGG) and the PCR were used to generate a 638 bp BamHI/NotI fragment that was cloned into the BamHI/NotI sites of pCDNAFlag. Subsequently, a 2.2-kb NotI fragment from the FOG-2 cDNA (2526–4770 bp) was cloned into the NotI site of FOG-2 cDNA (735–1151) primers (5'-CCGCCTGACGACGAGCATGTC and 5'-GGTCAACTGACGAGCATGTC) and the PCR were used to generate a 1.3-kb fragment from the FOG-2 cDNA (2478–3777 bp) that was inserted into the BamHI/XhoI site of pCDNAFlag. FOG-2 (752–1151), a 3.5-kb BamHI/XhoI fragment of the FOG-2 cDNA (122–2472 bp) was cloned into the BamHI/EcoRV sites of pCDNA3. FOG-2 (1–733)/Gal4, FOG-2(2–225)/Gal4, FOG-2(2–225)/GAL4, FOG-2(3–45)/Gal4, and FOG-2(2–247)/Gal4 were constructed by cloning N-terminal fragments of the FOG-2 cDNA 3' to the GAL4 DNA binding domain in the pCM vector (CLONTECH). FOG-2 (290–688) primers (5'-CCGGATCCCATGCGAGTTGACGAGG and 5'-GGTTTCTGCTTCGCTC) were used to generate a 883-bp fragment of the FOG-2 cDNA (132–1015 bp) that was cloned into the BamHI/XhoI sites of pcDNA3. FOG-2 (1–312)/Gal4, FOG-2 (1–125)/Gal4, FOG-2 (1–45)/Gal4, FOG-2 (16–195)/Gal4, FOG-2 (185–240)/Gal4, and FOG-2 (241–312)/Gal4 were constructed by cloning BglII fragments from the FOG-2 cDNA 5' to the GAL4 DNA binding domain in the pCM vector (CLONTECH). The PCR were used to generate a 1.1-kb fragment from the FOG-2 cDNA (1140–2276 bp) that was subsequently inserted into the BamHI/XhoI sites of pCDNAFlag.

pCDNA3-CtBP-2 was generated by cloning a 500-bp EcoRI/XhoI fragment encoding the 5'-end of the murine CtBP-2 cDNA from an expressed sequence tag clone (GenBank3' accession no. AA433525) into the EcoRI/XhoI site of pCDNA3. Subsequently, a XhoI/XhoI fragment containing the 3'-end of the FOG-2 cDNA was cloned into the XhoI/XhoI site.

Transfections—NIH 3T3 fibroblasts were transfected using 5μg of DNA and the Superfect reagent (Qiagen, Valencia, CA) following the manufacturer's instructions. All transfections were done in triplicate and contained 200ng of pVRβ-gal, a reference plasmid containing the cytomegalovirus promoter driving expression of the LacZ gene (33). Cells were harvested 48h later and assayed for growth hormone or luciferase levels, β-galactosidase activity, and total protein content using commercially available kits (Nichols Institute, San Juan Capistrano, CA; Promega, Madison, WI; and Bio-Rad). Growth hormone and luciferase levels were normalized to β-galactosidase activity and total protein content to correct for variations in transfection and cell harvesting efficiencies.

Electrophoretic Mobility Shift Assays—NIH 3T3 fibroblasts were transfected with pcDNA3-GATA4, pcDNA3-FOG-2, or both; 48h after transfection cells were harvested, and nuclear extracts were prepared as described previously (33). The cardiac enhancer factor-1 oligonucleotide (5'-GGCCAGCTGAGATACAGG), which contains the GATA4 binding site from the cTnC enhancer (20, 35), was 32P-labeled with the Klenow fragment of DNA polymerase. This oligonucleotide was incubated in the presence of 30μg of nuclear extract for 20min at 4°C, and protein-DNA complexes were resolved by electrophoresis in 4% non-denaturing gels and visualized by autoradiography as described previously (16).

In Vitro Binding Assays—In vitro binding assays were performed as described previously (33). Briefly, DH5α bacteria were transformed with DNA expression constructs encoding GST-GATA4 or GST-CtBP-2 fusion proteins. Bacteria were grown to mid-log phase and induced with 1mM isopropyl-1-thio-β-D-galactopyranoside for 4h at 30°C. Bacteria were sonicated, and the resulting lysates were incubated with 30μl of a 50% slurry of glutathione-Sepharose for 1h at 4°C. Subsequently, the beads were washed four times with binding buffer, and 50μl of bound material was eluted with 50mM NaCl, 50mM Tris-HCl, pH 7.5, 0.1% NP-40, 0.5mM ZnSO4, 0.25% bovine serum albumin, and protease inhibitors), and 5–15μl of radiolabeled in vitro translated full-length or truncated FOG-2 proteins were added in a total volume of 700μl of binding buffer. After a 1h incubation at 4°C, the beads were again washed five times, and the bound proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

RESULTS

FOG-2 Represses GATA-dependent Transcription from Cardiac Promoters—We have shown previously that FOG-2 is a potent inhibitor of the GATA4-dependent transactivation of several cardiac-restricted promoters including those from the cTnC, BNP, and ANF genes (33). To determine if FOG-2 is capable of repressing transcription from cardiac promoters by the FOG-2 transcription factors, NIH 3T3 cells were transiently transfected with expression plasmids for GATA4, FOG-2, and Nkx2.5 and a growth hormone reporter plasmid containing 638bp of the rat ANF promoter that contains functionally important binding sites for both GATA4 and Nkx2.5 (Fig. 1). Consistent with previous reports, both GATA4 and Nkx2.5 alone transactivated the ANF promoter more than 150-fold. Yet, when coexpressed with FOG-2, both GATA4 and Nkx2.5 are repressed more than 90-fold by FOG-2 (Fig. 1). This repression is specific to the ANF promoter and does not affect the expression of other cardiac promoters such as cTnC, BNP, or ANF genes (33). To determine if FOG-2 is a potent inhibitor of the GATA4-dependent transactivation, we have identified a non-CtBP-2-dependent repression domain of FOG-2 located at the N terminus of the protein (aa 1 to 247). This domain is both necessary and sufficient for FOG-2-mediated repression of GATA4-dependent transcription and is functionally conserved in the N-terminal region of FOG. Furthermore, when fused to a Gal4 DNA binding domain, the N-terminal repressor domain of FOG-2 can repress transcription from an SV40 promoter juxtaposed to Gal4 DNA binding sites.
and Methods." Results are displayed as the mean ± S.E. (n = 3).

fold (3, 22, 25, 36). Moreover, co-transfection of 3T3 cells with both GATA4 and Nkx2.5 expression constructs resulted in the synergistic activation of this promoter by more than 1100-fold. Forced expression of FOG-2 repressed transactivation of the ANF promoter by GATA4 but had no effect on the Nkx2.5-mediated activation of this same promoter. Interestingly, expression of FOG-2 repressed the synergistic activation of the ANF promoter by Nkx2.5 and GATA4, reducing the transactivation of the ANF promoter to the level of activation seen with Nkx2.5 expression alone. These results demonstrated that FOG-2 represses GATA4-dependent but not Nkx2.5-dependent transcription from the ANF promoter and that this repressor function therefore does not reflect a generalized or nonspecific inhibition of ANF promoter activity.

**Single Amino Acid Substitutions in the N-terminal Zinc Finger of GATA4 Abolish FOG-2-mediated Transcriptional Repression**—Previous studies have identified naturally occurring mutations in the N-terminal zinc finger of the Drosophila GATA protein, Pannier, that abolish its ability to interact with the FOG-2-related Drosophila protein, U-shaped (37). Similar mutations in the N-terminal zinc finger of GATA1 have been shown to abolish its interactions with FOG (38). To test the effects of such mutations on the promoter-FOG-2 interaction, we used PCR-based mutagenesis to generate single amino acid substitutions in the N-terminal zinc finger of GATA4 (D2 = Gly$^{233} \rightarrow$ Glu and D4 = Gly$^{215} \rightarrow$ Lys) (Fig. 2A). Both mutants demonstrated decreased binding to FOG-2 in GST binding assays (Fig. 2B). The D2 mutation reduced FOG-2 binding by 63 ± 9%, whereas the D4 mutation reduced FOG-2 binding by 95 ± 3%.

Electrophoretic mobility shift assays were used to determine if the D2 or D4 mutations had altered the DNA binding activity of GATA4. In these experiments, NIH 3T3 fibroblasts were transfected with expression constructs encoding wild-type or mutant GATA4. Forty-eight hours after transfection, cells were harvested, and nuclear extracts were assayed by electrophoretic mobility shift assay using a 32P-labeled oligonucleotide containing the cardiac enhancer factor-1 (CEF-1) GATA4 binding site from the proximal cTnC enhancer. D, GATA4 mutations attenuate FOG-2-mediated transcriptional repression. NIH 3T3 cells were transfected with a reporter construct containing the human growth hormone gene under the control of a 638-bp ANF promoter and expression constructs encoding FOG-2, GATA4, D2 GATA4, or D4 GATA4. Forty-eight hours after transfection, medium was assayed for human growth hormone, and the results were normalized for differences in transfection efficiencies. Results are displayed as the mean ± S.E. (n = 3).

**FIG. 1.** FOG-2 is a specific repressor of GATA4 transactivation. NIH 3T3 fibroblasts were transfected with a reporter construct containing the human growth hormone gene under control of the 638-bp ANF promoter and expression constructs encoding GATA4, FOG-2, or Nkx2.5 as indicated. Forty-eight hours after transfection, medium was assayed for human growth hormone, and the results were normalized for differences in transfection efficiencies as described under “Materials and Methods.” Results are displayed as the mean ± S.E. (n = 3).

**FIG. 2.** Single amino acid substitutions in the N-terminal zinc finger of GATA4 abrogate FOG-2-mediated transcriptional repression. A, schematic illustration of the GATA4 N-terminal zinc finger showing the four cysteines of the zinc finger coordinated to a zinc atom. The two amino acids altered by PCR-mediated mutagenesis (D2, Gly$^{233} \rightarrow$ Glu and D4, Glu$^{215} \rightarrow$ Lys) are shaded. B, GATA4 point mutations disrupt FOG-2 binding. In vitro binding assay using 32P-labeled, in vitro translated FOG-2 and bacterially expressed GST, GST-WT GATA4, GST-D2 GATA4, or GST-D4 GATA4 fusion proteins. C, GATA4 point mutations do not affect GATA4 DNA binding. Nuclear extracts prepared from NIH 3T3 cells transfected with expression plasmids encoding wild-type (WT) GATA4, D2 GATA4, or D4 GATA4 proteins were assayed by electrophoretic mobility shift assay with a 32P-labeled oligonucleotide containing the cardiac enhancer factor-1 (CEF-1) GATA4 binding site from the proximal cTnC enhancer. D, GATA4 mutations attenuate FOG-2-mediated transcriptional repression. NIH 3T3 cells were transfected with a reporter construct containing the human growth hormone gene under the control of a 638-bp ANF promoter and expression constructs encoding FOG-2, GATA4, D2 GATA4, or D4 GATA4. Forty-eight hours after transfection, medium was assayed for human growth hormone, and the results were normalized for differences in transfection efficiencies. Results are displayed as the mean ± S.E. (n = 3).
normalizes for differences in transfection efficiencies. As shown in Fig. 2D, both the D2 and D4 mutants were able to transactivate the BNP promoter to the same level as wild-type GATA4. Consistent with previous work (33), FOG-2 repressed the ability of wild-type GATA4 to activate this promoter by 10-fold. In contrast, activation of the BNP promoter by the D2 mutant, which displays moderately reduced FOG-2 binding, was only repressed 3-fold by overexpression of FOG-2. Transactivation by the D4 mutant, which fails to interact with FOG-2, was unaffected by FOG-2. These results suggested that the ability of FOG-2 to repress GATA4-mediated transcriptional activation is proportional to its ability to physically interact with the N-terminal zinc finger of GATA4. Moreover, they demonstrated that the susceptibility of GATA4 to FOG-2-dependent repression can be distinguished structurally from its DNA binding and transcriptional activation functions.

Multiple Zinc Fingers of FOG-2 Can Mediate Binding to GATA4—To more precisely identify the domains of FOG-2 required for GATA4 binding and transcriptional repression, we constructed a series of eukaryotic expression constructs containing N- and C-terminal truncations of FOG-2 (Fig. 3A). These constructs were used to generate 35S-labeled protein for in vitro GST binding reactions as shown in Fig. 3B. Deletion of aa 1–534, containing zinc fingers 1–4, did not affect the ability of FOG-2 to bind to GATA4 (Fig. 3B, lane 5). In contrast, the deletion of an additional 218 amino acids (aa 1–752) containing zinc fingers 5 and 6 resulted in a marked reduction in GATA4 binding (Fig. 3B, lane 6). Deletion of 418 amino acids from the C terminus of FOG-2 (aa 753–1151) also did not affect GATA4 binding (Fig. 3B, lane 7). However, the further deletion of zinc fingers 5 and 6 resulted in significantly decreased binding to GATA4 (Fig. 3B, lane 8). As expected, removal of all of the zinc fingers of FOG-2 resulted in the complete loss of GATA4 binding activity (Fig. 3B, lane 10). Interestingly, however, a peptide containing aa 1–312 of FOG-2 and including only zinc finger 1 retained GATA4 binding activity (Fig. 3B, lane 9) as did a peptide containing aa 1–506 of FOG-2. The decreased binding of aa 1–506 as compared with 1–312 may have reflected differences in the tertiary structures of these various artificial deletion constructs. However, the binding of both aa 1–312 and aa 1–506 was confirmed in the transcriptional repression assays shown in Fig. 3C (see below). Interestingly, fusion of the zinc finger 6 of FOG-2 to a peptide containing aa 1–247 from the N terminus of the protein also restored GATA4 binding activity (Fig. 3B, lane 11). In contrast, a peptide containing zinc fingers 2–5 (aa 290–668) was incapable of binding to GATA4 (Fig. 3B, lane 12). Taken together, these results demonstrated that either zinc finger 1 or zinc finger 6 of FOG-2 are sufficient to mediate binding to GATA4.

Identification of Functionally Conserved N-terminal Transcriptional Reppressor Domains in FOG-2 and FOG—To better define the protein domain(s) required for the transcriptional repressor activity of FOG-2, we used transient transfection assays to test the ability of the N- and C-terminal deletion mutants shown in Fig. 3A to repress GATA4-mediated transcriptional activation of the ANF promoter (Fig. 3C). Deletion of aa 1–226 abolished the ability of FOG-2 to repress GATA4-dependent transcription without altering its ability to physically interact with the N-terminal zinc finger of GATA4. In contrast, C-terminal deletions did not significantly affect the ability of FOG-2 to repress GATA4-dependent transcription until all of the zinc fingers were removed, resulting in the loss of GATA4 binding activity. From these experiments, we concluded that aa 1–226 of FOG-2 are required for its transcriptional repressor function.

Interestingly, expression of FOG-2 peptides lacking the repression domain (aa 1–226) but containing either aa 227–1151 or aa 290–668 resulted in superactivation of GATA4-dependent transcription (p < 0.02 and p < 0.03, respectively) as compared with the transfection lacking FOG-2 (Fig. 3C). This result suggested that these peptides that both bind GATA4 but lack the N-terminal repressor domain might be functioning as dominant-negative repressors (i.e., activators) in this assay. As such, they lent further support to the important role of aa 1–227 in the transcriptional repressor function of FOG-2 and suggested that 3T3 cells may express an endogenous FOG-like activity. This possibility is currently under investigation.

Previous studies have demonstrated that FOG is a potent repressor of GATA1-dependent transcription in transient transfection assays (27, 29). Although the zinc fingers of FOG
and FOG-2 are highly conserved, the N-terminal domains of the two proteins are only 27% identical at the amino acid level (Fig. 4A). Thus, it was of interest to determine 1) if FOG, like FOG-2, could repress the GATA4-dependent activation of cardiac promoters and 2) if so, whether the N-terminal domain of FOG was also required for its transcriptional repressor activity. As shown in Fig. 4B, full-length FOG repressed the GATA4-mediated activation of the ANF promoter in NIH 3T3 fibroblasts, or a deletion mutant of FOG lacking aa 1–230 (FOG-(230–995)). Forty-eight hours after transfection, media were assayed for human growth hormone and normalized for differences in transfection efficiencies. Results are displayed as the mean ± S.E. (n = 3).

Several experiments were performed to determine if the N-terminal repressor domain of FOG-2 was also sufficient to mediate repression of GATA4-dependent transcription. First, fusion of zinc finger 6 of FOG-2 to the N-terminal aa 1–247 was shown to simultaneously restore GATA4 binding and transcriptional repressor activity (Fig. 3C, aa 1–247 and 668–724). Similarly, the addition of zinc finger 1 to the N-terminal 247 amino acids also restored both GATA4 binding and transcriptional repression (Fig. 3C, aa 1–312). Finally, we fused the heterologous DNA binding domain of Gal4 to the putative transcriptional repression domain of FOG-2 (aa 1–312) and assayed the ability of this fusion protein to inhibit transcription from an SV40 promoter juxtaposed to four Gal4 DNA binding sites in the pSV40Gal4 Luciferase reporter plasmid. As shown in Fig. 5, the Gal4-FOG-2-(1–312) fusion protein was a potent inhibitor of SV40-dependent transcription. We further mapped this repression domain by fusing shorter fragments from this region of FOG-2 to the Gal4 DNA binding domain. As shown in Fig. 5, amino acids 1–45 of FOG-2 were sufficient to repress SV40-dependent transcription when fused to the Gal4 DNA binding domain. This effect was specific for this FOG-2 peptide because in control experiments expression of the Gal4 DNA binding domain alone or Gal4 fusion proteins containing aa 46–195, 183–240, or 241–312 of FOG-2 failed to repress SV40-dependent transcription (Fig. 5). Taken together, these experiments demonstrated that aa 1–247 of FOG-2 are both necessary and sufficient to repress GATA4- (and SV40-) dependent transcription so long as they are tethered to a domain that can bring this peptide into contiguity with the promoter/enhancer on the DNA.

Mapping of a Nuclear Localization Signal in FOG-2—We have shown previously that FOG-2 is localized in the nucleus of both primary rat neonatal cardiomyocytes and COS-7 cells transfected with a FOG-2 expression construct (33). To ensure that the loss of FOG-2 repressor activity observed following deletion of the N-terminal 247 amino acids did not simply reflect a defect in the nuclear localization of the mutant protein, we used the deletion mutants shown in Fig. 4A to map the putative nuclear localization signals of FOG-2. Indirect immunofluorescence was used to determine the subcellular localization of FOG-2, we transfected COS-7 fibroblasts with the N- and C-terminal truncation mutants of FOG-2 described in Fig. 3A. Indirect immunofluorescence was used to determine the subcellular localization of these truncation mutants with representative data shown in Fig. 6. Both full-length FOG-2 and N-terminal truncations containing aa 736–740 were localized exclusively to the nucleus. Thus, the loss of repressor function...
by N-terminal truncations lacking aa 1–247 was not because of the loss of nuclear localization. In contrast, removal of aa 736–740 resulted in redistribution of the resultant proteins to both the cytoplasm and the nucleus (Fig. 6, compare aa 735–745 and aa 736–740). Similarly, all C-terminal truncations lacking aa 736–740 were localized to both the nucleus and cytoplasm. However, each of these C-terminal deletion mutants repressed GATA4-dependent transcription (Fig. 7C). Thus, we concluded that the nuclear localization signal located between aa 736 and 740 is required for exclusive nuclear localization of FOG-2 and that the inability of the FOG-2 N-terminal truncations to repress GATA4 transactivation was not because of disrupted nuclear localization.

The Transcriptional Repressor CtBP-2 Binds to FOG-2 but Is Not Required for FOG-2-mediated Repression of GATA4-dependent Transcription—One mechanism by which FOG-2 might inhibit GATA4-dependent transcription is by recruiting one or more transcriptional co-repressors to the GATA4-FOG-2 complex. One such co-repressor is CtBP-2, which has been shown previously to bind to FOG via a consensus CtBP binding site (PIDLS) (29, 31). This putative CtBP binding site is also present in FOG-2 (aa 383–384). The ability of FOG-2 to bind CtBP-2 was tested using GST binding assays (Fig. 7B). GST-CtBP-2 specifically bound to full-length in vitro translated FOG-2 and to an N-terminal deletion of FOG-2 (aa 227–1151) that contains the PIDLS sequence but failed to bind to a C-terminal truncation (aa 1–733) that lacks this site. Thus, FOG-2, like FOG, is capable of binding CtBP-2.

To test the functional significance of the FOG-2-CtBP-2 interaction, NIH 3T3 fibroblasts were transfected with expression constructs encoding full-length or truncated FOG-2, CtBP-2, GATA4, and an ANF promoter reporter construct. Full-length FOG-2 repressed GATA4-dependent transcription from the ANF promoter by 7-fold (Fig. 7C). In contrast, expression of CtBP-2 repressed GATA4-mediated activation of the ANF promoter by only 20%. This modest repression by CtBP-2 occurred both in the presence and absence of FOG-2 indicating that it was not FOG-2-dependent. More importantly, deletion of the CtBP-2 binding site in FOG-2 (1–733), which abolished CtBP-2 binding (Fig. 7B), did not decrease the transcriptional repressor activity of FOG-2 (Fig. 7C; compare wild-type FOG-2 and FOG-2 (1–773)). From these experiments, we concluded that, at least in this experimental system, CtBP-2 is neither sufficient nor necessary for the transcriptional repressor function of FOG-2.

**DISCUSSION**

FOG-2 is a member of a family of evolutionarily conserved multizinc finger transcriptional modulators that includes the Drosophila protein, U-shaped, and the mammalian protein, FOG (26, 29, 30, 32–34). Each of the FOG proteins is known to interact with one or more GATA factors and to thereby co-activate or repress their transcriptional activities. Previous studies have demonstrated that FOG-2 is co-expressed with GATA4 in both embryonic and adult cardiomyocytes, can bind specifically to the N-terminal zinc finger of GATA4, and can function as a potent repressor of GATA4-dependent transcription in both fibroblasts and cardiomyocytes (29, 32–34). However, relatively little was previously understood about the molecular basis of the GATA4-FOG-2 interaction and the transcriptional repressor activity of FOG-2.

In the studies described in this report, we have investigated the mechanisms underlying FOG-2-mediated transcriptional repression of cardiac-restricted promoters. The results have
identified evolutionarily conserved modules within the FOG proteins that mediate their specific interactions with GATA transcription factors, program their nuclear localization, and determine their transcriptional repressor activities. Specifically, we have shown that 1) zinc fingers 1 and 6 are each capable of interacting with evolutionarily conserved motifs within the N-terminal zinc finger of all of the mammalian GATA proteins, 2) a structurally conserved binding site in FOG-2 (PIDLS) is required for its interaction with CtBP-2, and 3) a nuclear localization signal (RKRRK) between aa 736 and 740 of FOG-2 is required to program the exclusive nuclear targeting of the protein. Finally, and most importantly we have identified novel and functionally conserved domains at the N termini of FOG and FOG-2 that are both necessary and sufficient to repress GATA4-dependent transcriptional activation. Moreover, we have shown that this repression is independent of the transcriptional co-repressor CtBP-2. Taken together, these findings begin to define a set of evolutionarily conserved mechanisms by which FOG proteins repress GATA-dependent transcription in multiple cell lineages. As such, they have important implications for understanding the mechanism(s) by which FOG-2 modulates GATA4-dependent transcription in cardiomyocytes.

Previous studies suggested that the transcriptional repressor activity of FOG proteins is mediated by the binding of the transcriptional co-repressor CtBP-2 (29). In contrast, our results demonstrate that CtBP-2 binding by FOG-2 is neither necessary nor sufficient for the FOG-2-mediated repression of GATA4-dependent transcription. Instead, we have identified novel and functionally conserved repressor domains at the N termini of both FOG and FOG-2 that are necessary and sufficient for repression of GATA4-dependent transcription in vitro. Our finding of CtBP-2-independent transcriptional repression by FOG proteins is supported by in vitro mutagenesis studies showing that CtBP-2 binding is also not necessary for the FOG-mediated repression of GATA1-dependent transcription. Mutation of the CtBP-2 binding site in FOG resulted in only a 2-fold reduction in FOG-mediated repression of GATA1-dependent transcription. Thus, although both FOG and FOG-2 contain conserved and functional CtBP-2 binding sites, binding to CtBP-2 is not required for the ability of these proteins to repress either GATA1 or GATA4-dependent transcription. However, it should be emphasized that it remains possible that CtBP-2 enhances the transcriptional repressor activity of FOG-2 for GATA4 on some promoters in vivo. In this regard it will be of interest to mutate the CtBP-2 binding site of FOG-2 in mice and to compare their phenotype to that of the FOG-2 knockout mice that have recently been produced in our laboratory.

The finding that the N terminus of FOG-2 is both necessary and sufficient for repression of GATA4-dependent transcription and that this domain can repress transcription even when fused to the Gal4 DNA binding domain is consistent with a model in which the N-terminal region of FOG-2 either binds one or more co-repressors or interferes with the activity of transcriptional co-activators or basal transcription factors. The N-terminal repression domains of FOG and FOG-2 display only 27% sequence identity (Fig. 4A). However, these two peptides share several structural motifs. The N-terminal domains of both peptides are acidic and proline-rich. In addition, several regions of the peptides (e.g. aa 1–24 and 102–136) display significant sequence identity (Fig. 4A). These structurally similar motifs might represent shared binding domains for nuclear modulators of GATA-dependent transcription. Ongoing yeast two-hybrid studies designed to identify specific binding partners for the FOG repressor domains should allow us to distinguish these possibilities and to identify additional proteins that help to mediate the repressor activity of FOG-2.

The fact that a single amino acid substitution (Glu → Lys) in the N-terminal zinc finger of GATA4 can disrupt FOG-2 binding and repression without affecting the DNA binding or transcriptional activation functions of GATA4 suggests the possibility of genetically determining the function of the GATA4-FOG-2 interaction by knocking the Lys215 mutant of GATA4 into the GATA4 locus in mice. By comparing the phenotype of such mutant mice to those of the GATA4 and FOG-2 knockouts that have recently been produced in our laboratory (18), it should be possible to understand the GATA4-dependent and GATA4-independent functions of FOG-2 in the heart.

2 E. Svensson, G. Huggins, and J. Leiden, unpublished data.
In summary, the FOG-GATA interaction has been evolutionarily conserved from flies to man. The physiologic significance of this interaction is underscored by the evolutionary conservation of the two families of proteins and by the fact that in all cases studied to date, FOG and GATA family members are co-expressed in the same cell lineages. Taken together with previous results, our studies demonstrate that a conserved set of structural motifs in both proteins determine their interaction and regulate the FOG-mediated repression of GATA-dependent transcription. A complete understanding of the physiologic function of FOG-2 will await the phenotype of the FOG-2 knockout mice and the identification and better understanding of proteins that interact with FOG-2. By defining the functionally important domains of FOG-2, the results described in this report form the foundation for these ongoing studies of FOG-2-regulated cardiac gene expression.

Acknowledgments—We acknowledge R. Doherty for help in preparing this manuscript, T. Lis for help with the preparation of illustrations, and C. Bacani for technical assistance.

REFERENCES

1. Arceci, R. J., King, A. A., Simon, M. C., Orkin, S. H., and Wilson, D. B. (1993) Mol. Cell. Biol. 13, 2235–2246
2. Dorfman, D. M., Wilson, D. B., Bruns, G. A., and Orkin, S. H. (1992) J. Biol. Chem. 267, 1279–1285
3. Durocher, D., Charron, F., Warren, R., Schwartz, R. J., and Nemer, M. (1997) EMBO J. 16, 5687–5696
4. Evans, T., Reitman, M., and Felsenfeld, G. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5976–5980
5. Grepin, C., Dagnino, L., Robitaille, L., Haberstroh, L., Antakly, T., and Nemer, M. (1994) Mol. Cell. Biol. 14, 3115–3129
6. Ho, I. C., Vorhees, P., Marin, N., Oakley, B. K., Tsai, S. F., Orkin, S. H., and Leiden, J. M. (1991) EMBO J. 10, 1187–1192
7. Tsai, S. F., Martin, D. I., Zon, L. I., D'Andrea, A. D., Wong, G. G., and Orkin, S. H. (1989) Nature 339, 446–451
8. Fenney, L., Simon, M. C., Robertson, E., Klein, W. H., Tsai, S. F., D'Agati, V., Orkin, S. H., and Costantini, F. (1991) Nature 349, 257–260
9. Ting, C. N., Olson, M. C., Barton, K. P., and Leiden, J. M. (1996) Nature 384, 474–478
10. Tsai, P. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W., and Orkin, S. H. (1994) Nature 371, 221–226
11. Laverriere, A. C., MacNeill, C., Mueller, C., Poelmann, R. E., Burch, J. B., and Evans, T. (1994) J. Biol. Chem. 269, 23177–23184
12. Morrisey, E. E., Ip, H. S., Lu, M. M., and Parmacek, M. S. (1996) Dev. Biol. 177, 309–322
13. Morrisey, E. E., Ip, H. S., Tang, Z., Lu, M. M., and Parmacek, M. S. (1997) Dev. Biol. 183, 21–36
14. Wilson, D. B., Dorfman, D. M., and Orkin, S. H. (1990) Mol. Cell. Biol. 10, 4854–4862
15. Huang, W. Y., Bukerman, E., and Liew, C. C. (1995) Gene (Amst.) 153, 219–223
16. Ip, H. S., Wilson, D. B., Heikinheimo, M., Tang, Z., Ting, C. N., Simon, M. C., Leiden, J. M., and Parmacek, M. S. (1994) Mol. Cell. Biol. 14, 7517–26
17. Molkenst, J. D., Rayakolang, D. V., and Markham, B. E. (1994) Mol. Cell. Biol. 14, 4947–4957
18. Kuo, C. T., Morrisey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., Soudais, C., and Leiden, J. M. (1997) Genes Dev. 11, 1048–1060
19. Molkenst, J. D., Lin, Q., Duncan, S. A., and Olson, E. N. (1997) Genes Dev. 11, 1061–1072
20. Ko, L. J., and Engel, J. D. (1993) Mol. Cell. Biol. 13, 4011–4022
21. Merika, M., and Orkin, S. H. (1993) Mol. Cell. Biol. 13, 3999–4010
22. Lee, Y., Shiio, T., Kasahara, H., Jobe, S. M., Wiese, R. S., Markham, B. E., and Izumo, S. (1998) Mol. Cell. Biol. 18, 3120–3129
23. Merika, M., and Orkin, S. H. (1995) Mol. Cell. Biol. 15, 2437–2447
24. Molkenst, J. D., Lu, J. R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Grant, S. R., and Olson, E. N. (1998) Cell 93, 215–228
25. Sepulveda, J. L., Belaguli, N., Nigam, V., Chen, C. Y., Nemer, M., and Schwartz, R. J. (1996) Mol. Cell. Biol. 16, 3405–3415
26. Cubadda, Y., Heitzler, P., Ray, R. P., Bourouis, M., Ramain, P., Gelhart, W., Simpson, P., and Haenlin, M. (1997) Genes Dev. 11, 3083–3095
27. Fox, A. H., Kowalski, K., King, G. F., Mackay, J. P., and Crossley, M. (1998) J. Biol. Chem. 273, 33595–33603
28. Fox, A. H., Liew, C., Holmes, M., Kowalski, K., Mackay, J. P., and Crossley, M. (1999) EMBO J. 18, 2812–2822
29. Holmes, M., Turner, J., Fox, A., Chisholm, O., Crossley, M., and Chong, B. (1999) J. Biol. Chem. 274, 23491–23498
30. Tsang, A. P., Visvader, J. E., Turner, C. A., Fujiwara, Y., Yu, C. W., Weiss, M. J., Crossley, M., and Orkin, S. H. (1997) Cell 90, 109–119
31. Turner, J., and Crossley, M. (1998) EMBO J. 17, 5129–5140
32. Lu, J. R., McKinsey, T. A., Xu, H., Wang, D. Z., Richardson, J. A., and Olson, E. N. (1999) Mol. Cell. Biol. 19, 4495–4502
33. Svensson, E. C., Tufts, R. L., Polk, C. E., and Leiden, J. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9561–9566
34. Tevosian, S. G., Deconinck, A. E., Cantor, A. B., Rieff, H. I., Fujiwara, Y., Corfas, G., and Orkin, S. H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 950–955
35. Parmacek, M. S., Vora, A. J., Shen, T., Barr, E., Jung, F., and Leiden, J. M. (1992) Mol. Cell. Biol. 12, 1967–1976
36. Shiehima, I., Nomuro, I., Oka, T., Hiroi, Y., Mizuno, T., Takimoto, E., Monzen, K., Akawa, R., Akazawa, H., Yamasaki, T., Kudoh, S., and Yazaki, Y. (1999) J. Biol. Chem. 274, 8231–8239
37. Haenlin, M., Cubadda, Y., Blondeau, F., Heitzler, P., Lutz, Y., Simpson, P., and Ramain, P. (1997) Genes Dev. 11, 3096–3108
38. Crispino, J. D., Lodish, M. B., MacKay, J. P., and Orkin, S. H. (1999) Mol. Cell 3, 219–229