FOG-2 competes with GATA-4 for transcriptional coactivator p300 and represses hypertrophic responses in cardiac myocytes

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SUMMARY

A multizinc finger protein, FOG-2, associates with a cardiac transcription factor, GATA-4, and represses GATA-4-dependent transcription. GATA-4 is not only required for normal heart development, but is also involved in hypertrophic responses in cardiac myocytes. However, the effects of FOG-2 on these responses are unknown. The interaction of GATA-4 with a transcriptional coactivator p300 is required for its full transcriptional activity and the activation of the embryonic program during myocardial cell hypertrophy. We show here that exogenous FOG-2 represses phenylephrine-induced hypertrophic responses such as myofibrillar organization, increase in cell size and hypertrophy-associated gene transcription. Using immunoprecipitation-Western blotting, we demonstrate that FOG-2 physically interacted with p300 and reduced the binding of GATA-4 to p300. In addition, in COS7 cells, in which the function of endogenous p300 is disrupted, FOG-2 is unable to repress the GATA-4-depedent transcriptional activities. However, FOG-2 markedly repressed the p300-mediated increase in the DNA binding and transcriptional activities of GATA-4 in these cells. Similarly, FOG-2 inhibited phenylephrine-induced increase in the p300/GATA-4 interaction, the GATA-4/DNA binding and transcriptional activities of GATA-4-dependent promoters in cardiac myocytes as well. These findings demonstrate that FOG-2 represses hypertrophic responses in cardiac myocytes and that p300 is involved in these repressive effects.

Key Words: FOG-2, p300, transcription, hypertrophy, cardiac myocyte

Abbreviations used in this paper:
ET-1, endothelin-1
ANF, atrial natriuretic factor
MHC, myosin heavy chain
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
PE, phenylephrine
luc, luciferase
CAT, chloramphenicol acetyltransferase
EMSA, electrophoretic mobility shift assay
INTRODUCTION

The FOG (Friend of GATA) family comprises a novel class of multitype zinc finger nuclear proteins that serve as cofactors for GATA transcription factors in diverse developmental contexts. FOG-1 is coexpressed with GATA-1, -2, and -3 and involved in the differentiation of hematopoietic cell lineages (1). In contrast, FOG-2 is coexpressed with GATA-4, -5, and -6 in the heart through developmental and adult stages (2,3). FOG-2 physically interacts with the N-terminal zinc finger of GATA-4 and this interaction is required for normal morphogenesis of the heart during organogenesis. FOG-2−/− mice embryos are lethal at midgestation, and their hearts are characterized by thin ventricular myocardium, common atrioventricular canal, tetralogy of Fallot malformation, and absence of coronary vasculature (4,5). Disrupting the GATA-4/FOG-2 interaction by replacement of a single amino acid within GATA-4 results in embryonic lethality due to a remarkably similar constellation of cardiac defects (6). FOG-2 protein has been reported to repress GATA-4-dependent transcriptional activities in cardiac myocytes and in fibroblasts (3). However, the precise mechanisms by which FOG-2 perturbs the transcriptional activities of GATA-4 are unknown at present. A repressor domain of FOG-2 has been mapped to the N-terminal region, and shown to be distinct from the GATA-4 binding domain (7). Interestingly, FOG-2 does not exhibit repressive effects in certain cell types (3). Although FOG-2 associates with the potent transcriptional co-repressor C-terminal-binding-protein-2, repression by FOG-2 occurs even in the absence of this co-repressor (7). These findings suggest that alternative mechanisms are involved in FOG-2-mediated repression of the GATA-4 transcriptional activity.

GATA-4 not only plays a critical role in various stages of cardiogenesis, but also is one of the transcriptional factors that regulate hypertrophic responses in cardiac myocytes (8,9). During myocardial cell hypertrophy, activation of MEK-1/ERK1/2 results in the phosphorylation of serine residues in GATA-4, which enhances its transcriptional potency. Overexpression of GATA-4 in the heart is sufficient to induce cardiac hypertrophy in vivo, while expression of a dominant-negative form of GATA-4 in cardiac myocytes blocks agonist-induced hypertrophy (10). While FOG-2 can repress GATA-4-dependent transcriptional activities, the effects of exogenous FOG-2 on myocardial cell hypertrophy are unknown at present.
The full transcriptional activity of GATA-4 requires its interaction with an adenovirus associated protein, p300 (11,12). p300 acts as a transcriptional co-activator of many DNA binding factors and mediates the interaction of these factors with components of the basal transcriptional complex (13). p300+/− mouse embryos show abnormal heart development characterized by defects in cardiac muscle differentiation and in trabeculation, which leads to embryonic death (14). In the hearts of these mice, the expression of cardiac muscle structural proteins, such as myosin heavy chain and α-actin, is clearly reduced. These findings are compatible with in vitro data showing that p300 is required for cardiac-specific transcription and for differentiation of cardiac myocytes (15-18). p300 protein also serves as a cofactor of hypertrophy-responsive transcription factors such as MEF-2 and AP-1 as well as GATA-4 and involved in the activation of the embryonic gene program during myocardial cell hypertrophy (11,19-21). Since the zinc finger domains of GATA-4 are required for its interaction with p300, we have hypothesized that a multizinc finger protein, FOG-2, may also associate with p300. If so, considering the limiting amounts of p300 in cardiac nuclei, it might be possible that FOG-2/p300 association affects the binding of GATA-4 to p300.

For these reasons, in the present study we investigated whether FOG-2 can repress hypertrophic responses in cardiac myocytes, and if so, whether p300 is involved in this process. We show here that exogenous FOG-2 represses myocardial cell hypertrophy as well as GATA-4-dependent transcription. In addition, we provide the first evidence that FOG-2 interacts with p300 and competes with GATA-4 for binding p300, which might be one of the mechanisms by which FOG-2 represses GATA-4-dependent transcription in the presence of p300.

**MATERIALS AND METHODS**

**Cell Culture and Transfection**

Primary neonatal rat cardiac myocytes were prepared as previously described (15). Cardiac myocytes were transfected with DNA using LipofectAMINE PLUS (Invitrogen Life Technologies) as described previously. Luc and CAT activities were determined in the same cell lysate as described previously (15,22).
COS7 cells (African green monkey kidney cells) were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. The cells were washed twice with serum-free medium and then transfected with DNA using LipofectAMINE (Invitrogen Life Technologies) as described previously (12).

**Plasmid Constructs**

Expression vectors pwtFOG-2, pGATA-4, pCMVβ-gal and pCMVp300 contain the cytomegalovirus promoter/enhancer fused to murine FOG-2 cDNA, murine GATA-4 cDNA, β-galactosidase cDNA, or a full-length human p300 cDNA, respectively (9,15). The expression vector pwtFOG-2 was a generous gift from Dr. Jeffrey M. Leiden (University of Chicago, Chicago, IL) (2). The expression vector p(1-247)FOG-2 encodes FOG-2 amino acid sequences 1-247 that lacks GATA-4-binding zinc fingers and was kindly donated by Dr. Eric C. Svensson (Harvard University Medical School, Boston, MA). pCMVp300 was a gift from Dr. Richard Eckner (University of Zurich, Zurich, Switzerland) The plasmid construct pET-CAT contains the transcription start site-proximal 204-bp rat ET-1 promoter sequence fused to the bacterial CAT gene (23). In pmutGATA-ET-CAT, a GATA-4 binding site located at sequence –136 to –131 was mutated in the context of the 204-bp rat ET-1 promoter (23). pANFluc consists of the firefly luciferase (luc) cDNA driven by a 131-bp rat ANF promoter sequence (12). pRSVluc and pRSVCAT contain luc and CAT cDNAs respectively, driven by Rous sarcoma virus (RSV) long terminal repeat sequences (15). Plasmids were purified by anion exchange chromatography (QIAGEN), quantified by measurement of the OD260, and examined by electrophoresis on agarose gels and staining with ethidium bromide prior to use.

**Immunoprecipitation and Western blotting**

Nuclear extracts were prepared from COS7 cells or cardiac myocytes as described previously (15). Two hundreds micrograms of the extracts were precleared with 20 µl of 50% protein G-Sepharose slurry (Amersham Biosciences), and then immunoprecipitated using mouse monoclonal antibodies against human p300 (a mixture of RW109, RW105, and RW128 antibodies, Upstate Biotechnology), goat polyclonal anti-FOG-2 antibody (cat # sc-9365; Santa Cruz Biotechnology), rabbit polyclonal anti-GATA-4 antibody (cat # sc-9053; Santa Cruz Biotechnology) or normal rabbit IgG...
(Jackson ImmunoResearch Laboratories) in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) supplemented with 1 mM phenylmethylsulfonyl fluoride, and 1.0 µg/ml pepstatin A and 1.0 µg/ml aprotinin at 4°C overnight. Immune complexes were then collected by incubation with 15 µl of 50% Protein G-Sepharose preabsorbed with 1.0 g/ml bovine serum albumin at 4°C for 2 hours. The beads were washed three times in the lysis buffer, and subsequently three times in phosphate-buffered saline. Immune complexes were eluted by boiling for 5 minutes with 20 µl of 2×SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 10% -mercaptoethanol, 20% glycerol, 0.002% bromophenol blue), and resolved by SDS-polyacrylamide electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes (Hybond™ ECL™; Amersham Biosciences), reacted with the anti-p300 antibody, and subsequently detected using horseradish peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories). Signals were detected using an enhanced chemiluminescence Western blotting detection system (Amersham Biosciences) according to the manufacture’s instructions. Antibodies were stripped using 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-ME at 60°C for 30 minutes. The membrane was reprobed with goat polyclonal anti-GATA-4 antibody (cat # sc-1237; Santa Cruz Biotechnology).

**Immunohistochemistry and Measurement of Cell Surface Area**

The cardiac myocytes were grown in flask-style chambers on glass slides (Nalgen Nunc). The cells were fixed with 3% formaldehyde in phosphate-buffered saline for 15 minutes at room temperature. Immunocytochemical staining for β-MHC was performed using the indirect immunoperoxidase method as previously described (24).

A total of 40 myocardial fibers were selected randomly from cardiac myocytes stained with anti-β-MHC antibody, and the surface area of these cells were measured semiautomatically with the aid of an image analyzer (Image Pro-Plus) as previously described (24).

**RNA analysis**

Northern blot analysis of 10 µg of total RNA was performed as described previously (15). An isoform-specific antisense deoxyoligonucleotide complementary
to nucleotides 5846-5869 of the rat 3'-untranslated region (25) was used to detect β-MHC mRNA as described previously (15). To detect ANF mRNA, we used a 202-bp rat ANF cDNA probe obtained by reverse transcriptase-polymerase chain reaction (26). As controls, blots were also hybridized with rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (nucleotides 170-577) (27). To detect prepro-ET-1 and GAPDH mRNAs, we carried out reverse transcriptase (RT) PCR. Sequences of primers and precise conditions for RT-PCR were described previously (24). Signals of mRNAs were quantified by densitometry using NIH image 1.61, and the levels of β-MHC, ANF and prepro-ET-1 mRNAs were normalized relative to those of GAPDH mRNA.

**Electrophoretic Mobility Shift Assays (EMSAs)**

Double-stranded oligonucleotides for GATA-4 were designed based on the rat ET-1 upstream sequence that contains the GATA motif. The sequence of the sense strand of this oligonucleotide was as follows:

5'-CCTCTAGAGCCGGGTCTTA TCTCCGGCTGCACGTTGC-3'. Oligonucleotides were synthesized by Kurabo Inc. and purified by PAGE. Sp-1 consensus double-stranded oligonucleotides were purchased from Santa Cruz Biochemistry (cat # sc-2502)

EMSAs were carried out as described previously (9) at 4°C for 30 minutes in 20 µl reaction mixtures containing 7.5 µg of nuclear extract, 0.25 ng (>20,000 cpm) of radiolabeled double-stranded oligonucleotide, 500 ng of poly (dI-dC), 4 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM dithiothreitol, 37.5 mM KCl, and 4% Ficoll 400. For cold competition experiments, a 100-fold molar excess of unlabeled competitor oligonucleotide was included in the binding reaction mixture. Protein-DNA complexes were resolved by 4% nondenaturing PAGE in 0.25 × Tris-borate-EDTA at 4°C.

**Statistical analysis**

Data are presented as means ± standard error. Statistical comparisons were performed using unpaired two-tailed Student’s t tests or ANOVA with Scheffe’s test where appropriate, with a probability value <0.05 taken to indicate significance.
RESULTS

FOG-2 represses hypertrophic responses in cardiac myocytes

To examine the effects of exogenous FOG-2 on hypertrophic responses in cardiac myocytes, primary cardiac myocytes prepared from neonatal rats were transfected with an expression vector encoding β-gal (pCMVβ-gal), wild-type FOG-2 (pwtFOG-2) or mutant FOG-2 unable to bind GATA-4 (p(1-247)FOG-2). These cells were subsequently stimulated with saline or 10^{-5} M phenylephrine (PE), an α1-adrenergic agonist, for 48 hours, and stained with an antibody against β-myosin heavy chain (β-MHC). As shown in Fig. 1A, cardiac myocytes stimulated with PE displayed increases in cell size and myofibrillar organization as compared with saline-treated cells. These PE-induced changes were inhibited by transfection of pwtFOG-2. As shown in Fig. 1B, the PE-induced increases in myocardial cell surface area were significantly inhibited by transfecting pwtFOG-2, but not by transfecting p(1-247)FOG-2 (compare lanes 4-6). However, wild-type FOG-2 or mutant FOG-2 alone did not affect myocardial cell surface area under the basal condition (i.e., in saline-treated cardiac myocytes)(compare lanes 1-3). These data suggest that FOG-2 specifically repressed PE-induced growth of cardiac myocytes.

To examine the FOG-2’s effects on expression of hypertrophy-associated genes such as β-MHC, atrial natriuretic factor (ANF) and ET-1, we performed Northern blotting RT-PCR in cardiac myocytes transfected with pwtFOG-2 or pCMVβ-gal. As shown in the top and middle panels of Figs. 2A, FOG-2 almost completely inhibited the PE-induced upregulation of the expression of the endogenous β-MHC and ANF genes (compare lanes 1, 3 and 4). However, FOG-2 alone did not affect the basal level of these transcripts (compare lanes 1 and 2). Neither PE treatment nor pwtFOG-2 transfection affected the expression of the ubiquitously expressed GAPDH mRNA (bottom panel). The expression levels of β-MHC and ANF mRNAs were quantified and normalized relative to those of GAPDH mRNA. As shown in Fig. 2B, the PE-induced relative expression levels of β-MHC and ANF mRNAs were reduced by pFOG-2 transfection nearly to their control levels. Expression of prepro-ET-1, a precursor form of ET-1, was semi-quantified by RT-PCR. As shown in Figure 2C,
stimulation of cardiac myocytes with PE increased prepro-ET-1 levels in pCMVβ-gal-transfected cells. Expression of FOG-2, but not that of (1-247)FOG-2, almost completely inhibited the PE-induced increase in prepro-ET-1 mRNA levels. Taken together, these data demonstrate that exogenous FOG-2 can selectively suppress the PE-induced hypertrophic responses in cardiac myocytes.

**FOG-2 interacts with p300, and inhibits the binding of GATA-4 to p300**

The full transcriptional activity of GATA-4 requires its interaction with an adenovirus associated protein, p300 (11,12). Since the zinc finger domains of GATA-4 are required for its interaction with p300, we speculated that a multizinc finger protein, FOG-2, may also associate with p300. Therefore, to investigate the physical interactions between FOG-2 and p300, we performed immunoprecipitation, followed by Western blotting. COS7 cells were transfected with pwtFOG-2 and pCMVp300. Nuclear extracts from these cells were subjected to immunoprecipitation with anti-p300 antibody, with anti-FOG-2 antibody, and with normal rabbit IgG as a negative control. These immunoprecipitates were then subjected to Western blotting using the anti-p300 antibody. As shown in Fig. 3, an interaction between FOG-2 and p300 was clearly observed even after extensive washing (upper panel, lane 2). The anti-p300 antibody was stripped, and then the membrane was reprobed with the anti-FOG-2 antibody. Similarly, an interaction between FOG-2 and p300 was observed (lower panel, lane 1). These findings demonstrate that FOG-2 physically interacts with p300.

To examine whether FOG-2 affects the physical association of GATA-4 with p300, COS7 cells were cotransfected with either pcDNA3, pwtFOG-2 or p(1-247)FOG-2, in addition to pCMVp300 and pGATA-4. Nuclear extracts from these cells were subjected to immunoprecipitation with anti-GATA-4 antibody, with control IgG as a negative control, and with anti-p300 antibody, followed by Western blotting using the anti-p300 antibody. As shown in the left top panel of Fig. 4A, an interaction between GATA-4 and p300 was observed in pcDNA3-containing nuclear extracts (lane 1), in agreement with previously reported findings (9,38). Notably, the interaction was reduced in wild-type FOG-2-containing extracts (lane 2) compared with pcDNA3-containing ones (lane 1). In accordance with the fact that (1-247)FOG-2 is unable to GATA-4, this mutant FOG-2 did not reduce the p300/GATA-4 interaction (lane 3) as the wild-type did (lane 2). The levels of p300 proteins immunoprecipitated
with the anti-p300 antibody were similar among these three groups of samples (Fig. 4A, left bottom panel). After the anti-p300 antibody was stripped, the membrane was reprobed with the anti-GATA-4 antibody. As shown in the right bottom panel of Fig. 4A, expression of the wild-type FOG-2 but not the mutant FOG-2 reduced the p300/GATA-4 interaction, similar with the results in the left top panel. As shown in Fig. 4B, the expression levels of p300 (top panel) and GATA-4 (middle panel) before immunoprecipitation were similar among the three groups of samples (lanes 1-3). The expression levels of the wild-type and the mutant FOG-2 were comparable as well (lanes 2 and 3). We performed these immunoprecipitation/Western blotting experiments at least four times and found the results to be reproducible. These findings demonstrate that the expression of FOG-2 interferes with the binding of GATA-4 to p300.

**FOG-2 suppresses the p300-mediated increase in GATA-4/DNA binding activities**

Endothelin-1 (ET-1) is a vasoconstrictive and growth-promoting peptide, whose expression in cardiac myocytes is markedly induced during their hypertrophic responses. Increased cardiac ET-1 transcription during hypertrophy requires a functional GATA site within the ET-1 promoter (26). To determine whether GATA-4/DNA binding is modulated by FOG-2 and exogenous p300, we carried out electrophoretic mobility shift assays (EMSAs). COS7 cells were cotransfected with pGATA-4 and one of pwtFOG-2, p(1-247)FOG-2 or pcDNA3 in combination with pCMVp300, as indicated. Nuclear extracts from these cells were subjected to EMSAs using the ET-1 GATA site as a probe. As shown in Fig. 5A, competition EMSAs revealed that a retarded band represented specific binding, as evidenced by the fact that it was competed out by an excess of unlabeled wild-type ET-1 GATA oligonucleotide (lane 2), but not by the same amount of an oligonucleotide containing a mutant ET-1 GATA site (lane 3). In addition, the retarded band was clearly supershifted by an anti-GATA-4 antibody (lane 5), but not by normal rabbit IgG (lane 4). As shown in Fig. 5B, in the absence of exogenous p300, cotransfection of pwtFOG-2 in addition to pGATA-4 did not inhibit the amount of GATA-4/DNA binding (compare lanes 1 and 2). Consistent with our recent report (38), artificial overexpression of p300 markedly increased the amount of GATA-4/DNA binding (compare lanes 1 and 3). Remarkably, the amount of GATA-4/DNA binding was decreased by the expression of wild-type
FOG-2 in addition to p300 (compare lanes 3 and 4). However, co-expression of (1-247)FOG-2, which lacks the GATA-4-binding domains, did not decrease the binding as markedly as wild-type FOG-2 (compare lanes 3-5). On the other hand, the expression of GATA-4 was similar under all conditions, as shown by Western blotting (Fig. 5C, lanes 1-5). These findings suggest that the presence of p300 is required for the FOG-2-mediated down-regulation of GATA-4/DNA binding activity.

FOG-2 reduces the PE-induced increase of the interaction between GATA-4 and p300 in cardiac myocytes

To determine whether FOG-2 affects the physical interaction between GATA-4 and p300 in PE-stimulated cardiac myocytes, we carried out immunoprecipitation Western blotting. Cardiac myocytes were transfected with pwtFOG-2 or pCMVβ-gal and subsequently treated with saline or PE (10^-5 M) for 48 hours. Nuclear extracts from these cells were subjected to immunoprecipitation with anti-GA TA-4 antibody or normal rabbit IgG, followed by Western blotting using anti-p300 antibody. As shown in the bottom two panels of Fig. 6, the cardiac expression levels of p300 before immunoprecipitation were not altered by the transfection of pwtFOG-2, but were markedly increased by PE stimulation. However, the levels of GATA-4 were constant among these four groups of samples. As shown in the top panel of Fig. 6, the complex between GATA-4 and p300 increased in nuclear extracts from PE-stimulated cardiac myocytes compared with those from saline-treated cells (compare lanes 1 and 3). Notably, expression of FOG-2 suppressed the PE-induced increase in the p300/GA TA-4 interaction (compare lanes 3 and 4). However, FOG-2 did not affect the interaction in the saline-treated state (compare lanes 1 and 2). The anti-p300 antibody was stripped, and then the membrane was reprobed with the anti-GA TA-4 antibody. As shown in the second from the top panel of Fig. 6, GATA-4 proteins were similarly immunoprecipitated by the anti-GA TA-4 antibody. These findings demonstrate that FOG-2 inhibits the PE-induced p300/GA TA-4 interaction in cardiac myocytes.

FOG-2 suppresses the PE-induced increase in the DNA binding of GATA-4 in cardiac myocytes

To determine whether FOG-2 modulates GATA-4/DNA binding in cardiac
myocytes, EMSAs were performed. Cardiac myocytes were transfected with pCMVβ-gal or pwtFOG-2, and subsequently treated with saline or PE (10^{-5} M) for 48 hours. Nuclear extracts from these cells were subjected to EMSAs using the ET-1 GATA site as a probe. As shown in Fig. 7A, a retarded band represented specific binding and contained a complex that specifically immunoreacted with anti-GATA-4 antibody. These data indicate that the retarded band mainly consists of the complex between the ET-1 GATA site and cardiac GATA-4. As shown in Fig. 7B, the amount of GATA-4/DNA binding significantly increased in nuclear extracts from PE-treated myocytes compared with those from saline-treated cells (compare lanes 1 and 3), consistent with previous reports (10,23). FOG-2 suppressed the PE-induced increase in the amount of GATA-4/DNA binding (compare lanes 3 and 4) but did not suppress the amount in the saline-treated state (compare lanes 1 and 2). On the other hand, the expression levels of GATA-4 were similar among the four groups of samples (Fig. 7B, lower panel), and the amount of Sp-1/DNA binding was not changed by PE stimulation or pFOG-2 transfection (Fig. 7C, lanes 1-4). The amount of GATA-4/DNA binding relative to that of Sp-1/DNA binding was quantified and is shown in Fig. 7D. These findings indicate that FOG-2 suppresses the PE-induced upregulation of GATA-4/DNA binding in cardiac myocytes.

A p300 protein is involved in the FOG-2-mediated repression of GATA-4 transcriptional activity.

Next, we examined whether FOG-2 would repress the PE-induced activation of the ET-1 and ANF promoters in cardiac myocytes, and if so, whether overexpression of p300 could prevent this repression. Both promoters contain functionally important GATA-4 binding sites (12,23). In agreement with the findings of previous studies (2,3,28), FOG-2 repressed the GATA-4-mediated transactivation of ET-1 and ANF promoters in NIH3T3 cells (data not shown). Then we transfected into cardiac myocytes a CAT cDNA driven by the 204-bp rat ET-1 promoter (pET-CAT, Fig. 8A) or a luc cDNA driven by the 131-bp rat ANF promoter (pANFluc, Fig. 8B), in combination with pwtFOG-2, pCMVp300, and pCMVβ-gal as indicated. Subsequently the transfected cells were treated with saline or PE (10^{-5} M) for 48 hours. As shown in Figs. 8A and C, FOG-2 almost completely inhibited the PE-induced activation of ET-1 and ANF promoters (compare lanes 3 and 4), while FOG-2 did not affect these promoter
activities in saline-treated myocytes (compare lanes 1 and 2). However, neither PE nor FOG-2 affected the activity of a mutant ET-1 promoter in which GATA-4 site is mutated (pmutGATA-ET-CAT, Fig. 8B). In contrast to the data of wild-type FOG-2, mutant FOG-2 unable to bind GATA-4 did not significantly inhibit the PE-induced activation of ET-1 and ANF promoters (compare lanes 3, 4 and 6 in Figs. 8A and C). Notably, overexpression of p300 prevented the FOG-2-mediated repression of these promoter activities (compare lanes 4 and 5). These findings suggest that FOG-2 specifically blocks the PE-induced activation of the ET-1 and ANF promoters in cardiac myocytes, and that an excessive amount of p300 protein reverses the repression.

To investigate whether functional p300 is required for the FOG-2-mediated repression of the GATA-4-dependent promoter activities, we performed reporter assays in COS7 cells, in which the function of endogenous p300 is disrupted (2,10,11). As shown in Fig. 9, consistent with the EMSA data, FOG-2 markedly repressed the GATA-4-dependent ET-1 promoter activity in the presence of exogenous functional p300 (compare lanes 7 and 8). In contrast, FOG-2 did not repress, but rather enhanced, the ET-1 promoter activity in the absence of exogenous p300 (compare lanes 3 and 4). We then examined whether FOG-2 could repress GATA-4-dependent ET-1 promoter activity in the presence of a larger amount of exogenous p300. Interestingly, FOG-2 could not repress the ET-1 promoter activity in the presence of a large amount of exogenous p300 (compare lanes 9 and 10). These results demonstrate that the FOG-2-mediated transcriptional repression requires the presence of an adequate amount of functional p300.

**DISCUSSION**

FOG-2 is a member of a family of evolutionarily conserved multitype zinc finger transcriptional modulators, and binds specifically to the N-terminal zinc finger of GATA-4. Previous studies demonstrated that FOG-2 functions as a potent repressor of GATA-4-dependent transcription (3,7,28). GATA-4 is known to be a mediator of myocardial cell hypertrophy as well as a key player in diverse developmental processes in the heart (8,9,29). Using an *in vitro* model of myocardial cell hypertrophy induced by α1-adrenergic agonist, PE, we showed here that wild-type FOG-2 represses...
hypertrophic responses such as myofibrillar organization and increases in cell size. FOG-2 also represses PE-induced activation of the ANF and ET-1 promoters, whose upregulation requires GATA-4 and is closely associated with hypertrophy (12,23). These repressive effects cannot be explained by artificial or toxic actions of FOG-2, since FOG-2 alone exhibited no effect on saline-treated cardiac myocytes. Thus, FOG-2 may specifically affect hypertrophy signaling pathways. The present study also demonstrated that FOG-2 repressed the PE-induced DNA binding of cardiac GATA-4. Our data are compatible with the fact that a dominant-negative form of GATA-4 blocks hypertrophic responses in cardiac myocytes (10). These findings suggest that FOG-2-dependent repression of hypertrophic responses is mediated, at least in part, by inhibition of GATA-4-dependent transcriptional pathways, although we cannot rule out the possibility of GATA-4-independent mechanisms.

An adenovirus E1A-associated protein, p300, is a transcriptional coactivator that interacts with diverse enhancer proteins (13). In cardiac myocytes, p300 protein serves as an integrator of hypertrophy-responsive transcriptional factors such as GATA-4, MEF-2, and AP-1, and these interactions are required for their full transcriptional activities (11,19-21). In fact, a deletion mutant of p300 lacking the GATA-4 interaction domain (C/H3 domain) blocks GATA-4-dependent transcription (11). The present study provides the first evidence that FOG-2 interacts with p300. Although we cannot totally rule out the possibility that this interaction is indirect and mediated through proteins in COS7 cells, these cells do not express any members of the GATA transcription factor family. It has been shown that both N- and C-terminal zinc finger domains of GATA-4 interact with the C/H3 domain of p300 (11). Therefore, it is possible that a multiple zinc finger protein, FOG-2 binds to the same region of p300. The present study also demonstrated that the presence of FOG-2 reduced the interaction between GATA-4 and p300. It has been suggested that competition occurs for limiting amounts of intracellular p300 and that this competition results in transcriptional repression. For example, p53 forms complexes with p300, reduces the amount of p300 available for AP-1 and inhibits p300-mediated transactivation of the AP-1-regulated promoter (30). Other examples in which competition for p300 results in transcriptional repression include STAT-2/NF-κB, Cited2/HIF-1α and MEF-2/Cabin1 (31,32). Since the interaction of GATA-4 with p300 is required for its full transcriptional activity, the competition for p300 might be one mechanism of
FOG-2-mediated repression of the GATA-4 transcriptional activity.

The DNA binding activity of GATA-4 is regulated in part at the post-translational level. Activation of ERK1/2 results in the phosphorylation of the serine residue in GATA-4 and increases its DNA binding activity (23,33). Another mode of GATA-4 post-translational modification is acetylation. In addition to its bridging and scaffolding function, p300 is endowed with intrinsic histone acetyltransferase activity and can acetylate the GATA family of proteins (13). Acetylation of GATA-1 by p300 increases their DNA binding activity and is involved in hematopoietic differentiation (34,35). Our recent study demonstrated that the direct binding of GATA-4 to p300 is required for the acetylation of GATA-4 and the increase in DNA binding (38). Compatible with these data, the present study demonstrated that p300 up-regulated the DNA binding activity of GATA-4. Notably, FOG-2 repressed the p300-mediated increase in the GATA-4 DNA binding activity. While we cannot totally rule out the possibility that FOG-2 physically masks the DNA binding domain of GATA-4, FOG-2 did not affect the GATA-4 DNA binding activity in the basal state. Since FOG-2 reduces the direct binding of GATA-4 to p300, it is possible that FOG-2 inhibits the p300-mediated acetylation of GATA-4. It has also been reported that the activity of p300 is regulated, in part, by its phosphorylation (1). Therefore, an additional possible mechanism of FOG-2-mediated reduction of the p300-induced GATA-4/DNA binding includes inhibition of the p300 phosphorylation. However, further studies are needed to elucidate the precise mechanism by which FOG-2 inhibits GATA-4/DNA binding.

FOG-2 has an N-terminal repressor domain, which is necessary and sufficient to repress GATA-4-dependent transcriptional activation. This repressor domain in FOG-2 is distinct from the N-terminal zinc finger domain that interacts with GATA-4 (7). However, FOG-2 does not exhibit repressive effects in certain cell types. For example, while FOG-2 inhibits GATA-4-mediated transcriptional activation of the α-MHC promoter in cardiac myocytes, FOG-2 activates GATA-4-dependent transcription of the same promoter in COS7 cells (24), in which the endogenous p300 function is impaired (3). Compatible with this, the present study demonstrated that FOG-2 did not repress, but rather activated, the GATA-4-dependent transcription in COS7 cells. However, in the presence of an adequate amount of p300 in these cells, FOG-2 markedly repressed the GATA-4-dependent transcriptional activity. These
findings demonstrate that adequate p300 function is required for FOG-2-dependent transcriptional repression. Furthermore, in the presence of a more excessive amount of p300, FOG-2 was unable to repress GATA-4-dependent transcriptional activities. These findings further support the idea that competition for p300 might be one mechanism for the repression.

The present study demonstrates that FOG-2 binds to p300 and reduces the p300/GATA-4 interaction. However, in contrast to adenovirus E1A protein, FOG-2 did not strongly bind to p300. In fact, FOG-2 did not repress the induction of p21 promoter activity by p53, another p300-dependent transcription factor (data not shown). Thus, unlike E1A, FOG-2 does not generally inhibit p300-dependent transcription. These findings suggest that FOG-2’s binding to p300 is not the only mechanism by which it represses transcription. It remains possible that both binding to GATA-4 and competition for p300 contribute to the FOG-2-mediated repression. Therefore, to elucidate the precise mechanisms by which FOG-2 represses GATA-4 transcriptional activities, further studies will be required, especially on the interactions of these proteins with other corepressors or coactivators.

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REFERENCES


FIGURE LEGENDS

Figure 1. FOG-2 represses the development of hypertrophy in cultured neonatal cardiac myocytes. Neonatal rat ventricular cardiac myocytes were tranfected with 0.1 µg of pwtFOG-2, p(1-247)FOG-2 or pCMVβ-gal as a control. Subsequently, these cells were treated with saline (SS) or PE (1.0 × 10^{-5} M) for 48 hours and subjected to immunohistochemistry. The primary antibody to β-MHC was stained with a secondary antibody conjugated with peroxidase (brown signals). A. Representative photographs. B. Cell surface area was measured as described under “Materials and Methods”. Values are means ± S.E. (µm^2). The data are from 40 cells in each group.

Figure 2. Northern blot analysis of the expression of endogenous ANF, β-MHC, and GAPDH genes. Neonatal rat ventricular cardiac myocytes were tranfected with 2.0 µg of either pwtFOG-2 or pCMVβ-gal, as indicated. Subsequently, the cells were treated with saline (SS) or PE (1.0 × 10^{-5} M) for 48 hours. Blots containing total RNA (10 µg) from these myocytes were sequentially hybridized with an isoform-specific antisense deoxyoligonucleotide complementary to rat β-MHC mRNA, with a rat ANF cDNA, and with a rat GAPDH cDNA. A. Representative photographs. B. Signals were quantified by densitometry using NIH image 1.62 and the levels of β-MHC and ANF mRNAs relative to the level of GAPDH mRNA were determined. The relative mRNA level in saline-treated cardiac myocytes transfected with pCMVβ-gal was set at 1.0. Values are the means ± S.E. from three independent experiments. C. RT-PCR analysis of the expression of endogenous preproET-1 and GAPDH genes was performed as previously described (24). Representative photographs of RT-PCR are shown.

Figure 3. FOG-2 interacts with p300. COS7 cells were transfected with 4.0 µg of pwtFOG-2 and 8.0 µg of pCMVp300. Nuclear extracts prepared from these cells were immunoprecipitated with the anti-p300 antibody (lane 1), with the anti-FOG-2 antibody (lane 2), and with control IgG (lane 3). After SDS-PAGE, the immunoprecipitates were subjected to Western blotting using the anti-p300 antibody (upper panel). The anti-p300 antibody was stripped and then the membrane was reprobed with the anti-FOG-2 antibody (lower panel).
Figure 4. FOG-2 reduces the binding of GATA-4 to p300.  A. COS7 cells were cotransfected with 2.0 µg of pwtFOG-2, p(1-247)FOG-2, or pcDNA3 in addition to 8.0 µg of pCMVp300 and 2.0 µg of pGATA-4, as indicated. Nuclear extracts prepared from these cells were immunoprecipitated (IP) with anti-GATA-4 antibody (top panels), control IgG (middle panels), and anti-p300 antibody (bottom panels). After SDS-PAGE, the immunoprecipitates were subjected to Western blotting using the anti-p300 antibody (left panels). The anti-p300 antibody was stripped and then the membrane was reprobed with anti-GATA-4 antibody (right panels).  B. Nuclear extracts before immunoprecipitation (IP) were also subjected to Western blotting using the anti-p300 antibody (top panel), anti-GATA-4 antibody (middle panel), and anti-FOG-2 antibody (bottom panel).

Figure 5. FOG-2 represses the p300-mediated increase in the GATA-4 DNA binding activity.  A and B. COS7 cells were transfected with 2.0 µg of pwtFOG-2, p(1-247)FOG-2 or pcDNA3, and 8.0 µg of either pCMVp300 or pcDNA3, in addition to pGATA-4. The total DNA content was equalized in each sample with pcDNA3. Nuclear extracts derived from these cells were probed with a radiolabeled oligonucleotide containing the ET-1 GATA site. The arrows indicate the complex corresponding to the GATA-specific interaction between the ET-1 GATA site and GATA-4.  A represents a competition study in which unlabeled competitor DNAs were present as indicated: wt, wild-type ET-1 GATA; mut, ET-1 GATA with a mutation. Equal amounts of normal rabbit IgG (lane 4) and anti-GATA-4 antibody (lane 5) were added to the binding mixture.  C. The same nuclear extracts as in (B) were subjected to Western blotting using the anti-GATA-4 antibody.

Figure 6. FOG-2 reduces the PE-induced increase of the interaction between GATA-4 and p300 in cardiac myocytes. Neonatal rat ventricular cardiac myocytes were transfected with 6.0 µg of either pwtFOG-2 or pCMVβ-gal, as indicated. Subsequently, the cells were treated with saline (SS) or PE (1.0 × 10⁻⁵ M) for 48 hours. Nuclear extracts prepared from these cells were immunoprecipitated (IP) with anti-GATA-4 antibody (lanes 1-4) and control IgG (lane 5). After SDS-PAGE, the immunoprecipitates were subjected to Western blotting using the anti-p300 antibody.
A-C. Cardiac myocytes were tranfected with 6.0 µg of pwtFOG-2 or pCMVβ-gal and treated with saline (SS) or PE (1.0×10^{-5} M) for 48 hours. Nuclear extracts from these cells were probed with a radiolabeled oligonucleotide containing the ET-1 GATA site (A and B) or the Sp-1 site (C). The arrows indicate the complex corresponding to the interaction between the ET-1-GATA-probe and GATA-4 (A and B), or the interaction between the Sp-1-probe and Sp-1 (C). A represents a competition study in which unlabeled competitor DNAs were present as indicated: (lane 3) wild-type ET-1 GATA (wt); (lane 2) ET-1 GATA with a mutation (mut). Equal amounts of normal rabbit IgG (lane 4) and anti-GATA-4 antibody (lane 5) were added to the binding mixture. **B. lower panel.** The nuclear extracts used for EMSA in B (upper panel) were subjected to Western blotting using the anti-GATA-4 antibody. **D.** The amount of GATA-4/DNA binding in B and that of Sp-1/DNA binding in C were quantified by densitometry using NIH image 1.62, and the relative amount of DNA binding (GATA-4/Sp-1) was determined in each sample. The amount in saline (SS)-treated cardiac myocytes transfected with pCMVβ-gal was set at 1.0 in each experiment. Values are the means ± S.E. of three independent experiments.

**Figure 8. FOG-2 represses PE-induced activation of the ET-1 and ANF promoter activities in cardiac myocytes.** Cardiac myocytes were tranfected with 1.0 µg of pET-1-CAT (A), pmutGATA-ET-CAT (B) or pANFluc (C) reporter and 0.1 µg of pRSVluc (A and B) or pRSVCAT (C) in the presence or the absence of 0.1 µg of pwtFOG-2 or p(1-247)FOG-2 and 1.0 µg of pCMVp300, as indicated. The total DNA content was equalized in each sample with pCMVβ-gal. These cells were subsequently treated with saline (SS) or PE (1.0×10^{-5} M). Forty-eight hours later, the cells were collected and the relative CAT activity (pET-CAT/pRSVluc in A and pmutGATA-ET-CAT/pRSVluc in B) or luc activity (pANFluc/pRSVCAT) (C) was determined. The relative reporter activity in saline-treated cardiac myocytes

The anti-p300 antibody was stripped and then the membrane was reprobed with the anti-GATA-4 antibody (second from top panel). Nuclear extracts before immunoprecipitation were also subjected to Western blotting using the anti-p300 antibody (second from bottom panel), and anti-GATA-4 antibody (bottom panel).

**Figure 7. FOG-2 suppresses the PE-induced increase in the DNA-binding activity of cardiac GATA-4.**
transfected with pCMVβ-gal was set at 1.0 in each experiment. The data shown are the means ± S.E. of two independent experiments, each performed in duplicate.

**Figure 9. FOG-2-mediated transcriptional repression requires the presence of an adequate amount of p300 protein.** COS7 cells were cotransfected with 0.8 µg of a pET-CAT reporter and 0.04 µg of pRSVluc in the presence or the absence of 0.8 µg of pGATA-4, 0.8 µg of pwtFOG-2, and 0.8 (+) or 2.0 µg (++) of pCMVp300, as indicated. The total DNA content was equalized in each sample with pCMVβ-gal. Forty-eight hours later, the relative CAT activities (CAT/luc) were determined. The results are expressed as fold activation of the normalized CAT activities (CAT/luc) relative to that produced by cotransfection with the control pCMVβ-gal. The data shown are the means ± S.E. of two independent experiments, each performed in duplicate. Lower panels; Expression of exogenous p300, GATA-4 and FOG-2 are examined by Western blotting.
**Fig. 2**

**A**

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>PE</th>
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<tbody>
<tr>
<td>FOG-2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>β-MHC</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>ANF</td>
<td></td>
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<tr>
<td>GAPDH</td>
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**B**

β-MHC

![Bar chart showing fold increase with p<0.01](chart)

ANF

![Bar chart showing fold increase with p<0.01](chart)

**C**

preproET-1

![Example of preproET-1 expression](chart)

GAPDH

![Example of GAPDH expression](chart)
Fig. 6

SS          PE

wt FOG-2    -  +  -  +  -

Lane 1 2 3 4 5

Blot: αp300

IP: αGATA-4  IgG

Blot: αGATA-4

IP: αGATA-4  IgG

Blot: αp300

Without IP

Blot: αGATA-4

Without IP
FOG-2 competes with GATA-4 for a transcriptional coactivator p300 and represses hypertrophic responses in cardiac myocytes

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