FOXC1 Transcriptional Regulation Is Mediated by N- and C-terminal Activation Domains and Contains a Phosphorylated Transcriptional Inhibitory Domain*

Received for publication, October 25, 2001, and in revised form, December 13, 2001
Published, JBC Papers In Press, January 8, 2002, DOI 10.1074/jbc.M110268200

Fred B. Berry‡‡, Ramsey A. Saleem¶¶, and Michael A. Walter‡¶**
From the Departments of §Ophthalmology and ¶¶Medical Genetics, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

Mutations in the FOXC1 gene result in Axenfeld-Rieger malformations of the anterior segment of the eye and lead to an increased susceptibility of glaucoma. To understand how the FOXC1 protein may function in contributing to these malformations, we identified functional regions in FOXC1 required for nuclear localization and transcriptional regulation. Two regions in the FOXC1 forkhead domain, one rich in basic amino acid residues, and a second, highly conserved among all FOX proteins, were necessary for nuclear localization of the FOXC1 protein. However, only the basic region was sufficient for nuclear localization. Two transcriptional activation domains were identified in the extreme N- and C-terminal regions of FOXC1. A transcription inhibitory domain was located at the central region of the protein. This region was able to reduce the trans-activation potential of the C-terminal activation domain, as well as the GAL4 activation domain. Lastly, we demonstrate that FOXC1 is a phosphoprotein, and a number of residues predicted to be phosphorylated were localized to the FOXC1 inhibitory domain. Removal of residues 215–366 resulted in a transcriptionally hyperactive FOXC1 protein, which displayed a reduced level of phosphorylation. These results indicate that FOXC1 is under complex regulatory control with multiple functional domains modulating FOXC1 transcriptional regulation.

FOX (Forkhead Box (1)) proteins are a family of transcription factors that contain an evolutionarily conserved 110-amino acid DNA-binding domain known as the forkhead domain (FHD). This DNA-binding domain was first identified as a region of homology between the Drosophila fork head protein (2) and the rat hepatocyte nuclear factor 3 protein (3). The FHD is composed of a variant of the helix-turn-helix motif, known as the winged-helix motif (4). Members of this family of transcription factors play key roles in development, including morphogenesis and cell fate specification (5). Recently a number of disease-causing mutations have been identified in human FOX genes. FOXC2 mutations are responsible for Hereditary Lymphedema-Distichiasis Syndrome (6). FOXL2 is mutated in patients with Blepharophimosis Ptosis/Epicanthus Inversus Syndrome (7). Thyroid agenesis has been linked to FOXE1 mutations (8). Speech disorders are linked to mutations in FOXP2 (9). Mutations in FOXC1 underlie Axenfeld-Rieger (AR) anterior segment malformations in the eye that result in a spectrum of glaucoma phenotypes in humans (10–13). Together these mutations indicate a vital role for FOX genes in disease and development.

In addition to its role in ocular development, FOXC1 is believed to be essential in the formation of mesoderm tissues. Foxc1 is responsible for the congenital hydrocephalus mutation in mice, which results in perinatal death with defects in the eye and skeletal system (14). Moreover, targeted deletions of Foxc1 in mice have demonstrated a crucial role for this protein in the formation of the kidney, eye, heart, bone, and cartilage (15–17). Furthermore, AR patients with FOXC1 mutations present with systematic findings, including dental dysgenesis and, in rare cases, cardiac defects (12, 18). These data suggest that FOXC1 is essential for the formation of mesoderm-derived tissues in vertebrates.

Our laboratory has previously determined that the disease-causing missense mutations in the FOXC1 FHD result in impaired DNA binding and reduced trans-activation of target genes (19). Little is known about FOXC1 functional domains outside of the forkhead box. To gain insight on how FOXC1 may contribute to the AR malformations and formation of mesoderm-derived structures, we sought to determine which regions of the protein are functionally important for nuclear localization and transcriptional activation. We find that FOXC1 is under complex regulatory control as multiple domains are required for the correct targeting of FOXC1 to the nucleus and for efficient activation of a FOXC1 transcriptional reporter gene.

EXPERIMENTAL PROCEDURES

Plasmids—The FOXC1 expression vector pcDNA4-FOXC1 has been reported previously (19). Deletions in the FHD were created by site-directed mutagenesis using the QuickChange mutagenesis kit (Stratagene) as described by the manufacturer. Mutagenic primers were designed to “loop out” the desired nucleotides from the FOXC1 plasmid. The introduction of the correct mutation was confirmed by sequencing, and the mutated region was subcloned back into pcDNA4-FOXC1. The mutagenic primers are listed as follows: A69–178: forward 5’-cgcgcctaccttctgggaacgccggagaagaggg-3’; reverse 5’-ctctcttcttgcaacctcagcag-3’; A199–337: forward 5’-cgcgcctaccttctgggaacgccggagaagaggg-3’; reverse 5’-ctctcttcttgcaacctcagcag-3’. The oligonucleotides correspond to the
coding sequence of the regions of the FOXC1 FHD were synthesized, annealed, and ligated into pEGFP. FOXC1 N- and C-terminal deletions were created by restriction digestion of pcDNA4-FOXC1. To create FOXC1 C-terminal deletions 1–475, 1–435, 1–366, and 1–215, the 3'-end of the pcDNA4-FOXC1 vector was digested with either KpnI, BsiXI, PstI, or Rsr II, respectively, end-filled with Klenow or T4 polymerase, and re-ligated into a blunt-ended XbaI site. FOXC1-(29–281) was created by subcloning an SpeI fragment of FOXC1 into the EcoRV site of pcDNA4C. FOXC1 digested with ApaI and XbaI was subcloned back into identical sites of FOXC1 (29–281) to create FOXC1-(29–553). FOXC1-(51–221) and FOXC1-(51–553) were created by a similar strategy with a NsiI fragment of FOXC1. Δ215–366 was created by digesting pcDNA4-FOXC1 with RsrII and PstI to remove the intervening sequence, end-filled with Klenow and T4 polymerase, respectively, and religated. The GALA DNA-binding domain vector, pM1 (CLONTECH), was modified by PCR to create the vector pM1-B with a multiple cloning site in-frame with the FOXC1 cDNA. GALA + FOXC1 fusions were constructed by restriction digestion of corresponding fragments of the FOXC1 cDNA and subcloning into pM1 or pM1-B vectors. Insertion of FOXC1 cDNAs into the correct reading frame was verified by sequencing.

Cell Culture—HeLa and COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Indirect Immunofluorescence—FOXC1 immunofluorescence was performed as described previously (19). For GFP fluorescence, HeLa cells were grown on coverslips and transfected with GFP vectors. Twenty-four hours following transfection, the coverslips were harvested onto microscope slides.

Trans-activation Assays—FOXC1 trans-activation assays were performed as described previously (19). For GALA trans-activation studies, COS-7 cells were plated at a density of 1 × 10⁵ cells/ml into six-well tissue culture plates 24 h prior to transfection. Twenty-eight hours after transfection, the cells were harvested and analyzed for luciferase activity per the manufacturer’s directions (Promega). Each transfection was performed in duplicate and replicated at least three times.

Immunoblotting—COS cells transfected with 5 μg of FOXC1 expression vectors or HeLa cells extracts transfected with 2 μg of FOXC1 expression vectors were resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Proteins were detected by immunoblotting with an anti-Xpress antibody (Invitrogen) at a concentration of 0.2 μg/ml and visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

RESULTS

The FOXC1 FHD Contains Two Independent Nuclear Localization Signals—The nuclear localization signals (NLSs) for FOX family members FOXA2 and FOXF2 are found in the FHDs (20, 21). To test whether such information resides in the FOXC1 FHD, mutations were created in the FOXC1 protein that either deleted the entire FHD or regions at the N- and C-terminal boundaries of the FHD. The cellular localization of these proteins was visualized by indirect immunofluorescence against vector-encoded Xpress epitope. The full-length FOXC1 protein was localized exclusively to the nucleus, whereas FOXC1 Δ69–178, which lacks the entire FHD, displayed an increased cytoplasmic localization (Fig. 1), indicating that the FHD contains information necessary for nuclear localization of FOXC1. Analysis of the amino acid sequence of the FOXC1 FHD revealed a span of basic amino acids at positions 168–176 that may serve as a potential NLS. Deletion of these amino acids from FOXC1 (Δ168–176) resulted in localization to both the cytoplasm and nucleus (Fig. 1). Deletion of residues 78–93 displayed a mixture of cytoplasmic and nuclear localization, whereas FOXC1 Δ78–93 resulted in localization to both the cytoplasm and nucleus (Fig. 1). Deletion of residues 78–93 resulted in a predominantly mixed cytoplasmic and nuclear localization similar to that observed when the entire FHD was removed. Therefore, two regions in the FHD are required for correct FOXC1 nuclear localization.

The two putative FOXC1 NLS domains were fused to green fluorescence protein (GFP) to determine if these signals were sufficient for nuclear localization. FOXC1 fused to GFP resulted in a localization of the GFP signal to the nuclei of HeLa cells (Fig. 2B). Expression of GFP + 77–93 produced a signal that was localized to both the cytoplasm and the nucleus of HeLa cells (Fig. 2C) indicating that this portion of FOXC1 is not localized.
sufficient for nuclear localization and therefore does not contain the FOXC1 NLS. Expression of GFP_{168-176}, on the other hand, led to the accumulation of GFP exclusively in the nucleus (Fig. 2D). Moreover, the GFP signal produced by this chimeric protein was enriched in a subnuclear domain resembling that of the nucleolus (Fig. 2D). These data indicate that only residues 168–176 was sufficient to localize GFP to the nucleus and may constitute the FOXC1 NLS domain.

**Transcription Activation by FOXC1 Is Mediated by N- and C-terminal Activation Domains**—To identify transcription activation domains in the FOXC1 protein, a series of N and C-terminal deletions flanking the FHD were created and tested for the ability to activate expression. At present, there are no known transcriptional targets of FOXC1, therefore a luciferase reporter that contains six FOXC1 binding sites (19) was used to monitor transcriptional activation. Transfection of HeLa cells with the full-length FOXC1-(1–553) cDNA resulted in an approximate 10-fold induction of luciferase activity compared with the empty expression vector alone (Fig. 3A). When N-terminal amino acids 1–29 or 1–51 were deleted from FOXC1, activation of the luciferase reporter was reduced by 50 and 55%, respectively, indicating that residues in the N terminus are required for full activation. In addition, removal of C-terminal residues also impaired activation as FOXC1-(1–435) reduced the luciferase reporter by 58%. These results indicate that FOXC1 contains segments from amino acids 1–51 in the N terminus, and from 435–553 in the C-terminal domain that are both required for efficient activation of the FOXC1 reporter. Expression of a FOXC1 protein that lacks both N- and C-terminal regions (FOXC1-(51–221)) was incapable of activating luciferase expression above levels observed with the empty expression vector. Interestingly, FOXC1-(1–366) activated luciferase expression to levels nearly equivalent to full-length FOXC1, while FOXC1-(1–215) activated luciferase to levels exceeding those observed for full-length FOXC1 (Fig. 3A). These results suggest that further deletion of the C terminus removed an inhibitory domain located between residues 215 and 365 that led to an enhanced activation of the luciferase reporter by the N-terminal *trans*-activation domain.

We verified that all FOXC1 deletion constructs used in *trans*-activation assays were capable of producing the correct protein at near equivalent amounts (Fig. 3B). In addition each FOXC1 deletion was capable of binding to the FOXC1 binding site by electrophoretic mobility shift assays (data not shown) indicat-
ing that the loss of transcriptional activation observed was the result of removing activator domains and not from the production of a protein incapable of binding DNA. These data are not surprising, because all FOXC1 deletions retain an intact FHD and previous reports indicate that expression of the FHD alone is sufficient to bind DNA in a sequence-specific manner (22).

The FOXC1 N- and C-terminal activation domains were fused in-frame to the GAL4 DNA-binding domain (DB), and their ability to activate transcription was tested. Full-length FOXC1 fused to the GAL4 DB activated the GAL4 luciferase reporter by 10-fold compared with the GAL4 DB alone (Fig. 4). Expression of N-terminal FOXC1 residues 1–30 and 1–65 were capable of activating luciferase expression to levels equivalent to full-length FOXC1, suggesting that the N-terminal regions are sufficient to activate transcription. GAL4+435–553 was over 10 times more active than full-length FOXC1 and 100-fold more active than base-line luciferase activity indicating that the principal C-terminal activation domain lies in these residues. The activation mediated by GAL4+366–553 and GAL4+215–553 was considerably lower than that of GAL4+435–553 indicating that residues between 215 and 434 may constrain the activation domain present in residues 435–553.

FOXC1 Is a Phosphoprotein—Extracts of COS-7 cells transfected with the FOXC1 cDNA were analyzed for protein expression. FOXC1 migrated as multiple immunoreactive bands on an SDS-polyacrylamide gel (Fig. 5A). The presence of the higher molecular weight proteins was reduced when extracts were incubated with calf intestinal alkaline phosphatase (CIP), indicating that the multiple FOXC1 bands were a result of phosphorylation. Inhibition of CIP activity by sodium vanadate (VO₃) and EDTA, retained the higher mobility FOXC1 bands. To determine if the phosphorylation of FOXC1 led to a protein conformation change, we performed a limited trypsin digest of FOXC1. The addition of CIP rendered FOXC1 more sensitive to the trypsin digestion than untreated FOXC1 or FOXC1 treated with CIP and VO₃ as indicated by the disappearance of the full-length FOXC1 band and the appearance of smaller tryptic fragments (Fig. 5B). These data suggest that dephosphorylation of FOXC1 increased accessibility for trypsin.

The phosphorylated regions of FOXC1 were mapped by Western analysis of the FOXC1 deletion constructs described in Fig. 3. FOXC1 residues 1–215 produced only a single immunoreactive band, whereas FOXC1(1–366) was detected as multiple FOXC1 bands (Fig. 5C). The migration of these additional bands was reduced by incubation with CIP. Additional FOXC1 immunoreactive bands were also observed for FOXC1-(1–466) and -(1–475) (data not shown). Based on these expression patterns we conclude that the phosphorylation of FOXC1 resulting in altered migration occurs between residues 215 and 366. When these residues were removed (FOXC1Δ215–366), the protein migrated as a single immunoreactive band, insensitive to CIP treatment (Fig. 5C). In addition, FOXC1Δ215–366 activated the FOXC1 reporter—2-fold higher than full-length FOXC1 (Fig. 5D). Amino acids 215–366 of FOXC1 contain phosphorylated residues and may serve to negatively modulate transcriptional activation by FOXC1.

We next investigated whether residues 215–366 contained repressor activity and could impair other trans-activation domains. When residues 215–366 fused to GAL4 DB were transfected with a GAL4-luciferase reporter under the control of the constitutively active SV40 promoter, luciferase levels were equivalent to levels observed with the GAL4 DB alone (Fig. 5E). The GAL4 activator domain (AD) fused to the GAL4 DB led to a robust activation of the GAL4-SV40-luc reporter. However, when GAL4+215–366 was fused to GAL4 AD, luciferase levels were markedly reduced (Fig. 5E). Therefore, FOXC1-(215–366) does not contain intrinsic transcriptional repressor activity, rather these residues may serve to inhibit the ability of activation domains to stimulate transcription.

**DISCUSSION**

In this report we describe the molecular dissection of FOXC1 to identify important functional regions required for nuclear localization and regulation of transcription. Two regions in the FOXC1 FHD were required for correct localization of FOXC1 into the nucleus (Fig. 6). Previous studies had identified that regions at the N- and C-terminal boundary of the FHD were critical for proper nuclear localization FOXA2 and FOXP2 (20, 24). The first region required for FOXC1 nuclear localization spans from residues 77 to 93 in the FHD and the removal of these residues prevented the nuclear accumulation of FOXC1. This domain was not sufficient for nuclear localization, because its attachment to GFP led to a mixed cytoplasmic and nuclear accumulation of the fusion protein (Fig. 2C). The amino acid sequence present in this region does not match any typical NLS sequence.
motif, however, these residues are some of the most highly conserved residues found in all FHDs (5). Thus this region is likely not an NLS but rather may serve as a NLS accessory domain, because it was necessary for proper nuclear localization of FOXC1. The second region contains a basic stretch of amino acids at position 169RRRRRFKK176 at the C-terminal end of the FHD that is similar to NLSs found in a number of HOX proteins (23). Like FOXC1, the C-terminal NLS in FOXA2 and FOXF2 is also rich in basic amino acid residues (20, 24). The basic region was necessary for FOXC1 nuclear localization and was sufficient to localize GFP to the nucleus. Although it was thought that both regions constituted a FOX NLS, our data suggests that only the C-terminal region of the FHD, rich in basic residues, represents a bona fide NLS.

FOXC1 contains N- and C-terminal transcription activation domains at positions 1–51 and 466–553, respectively (Fig. 6). Both regions are required for full activation of a FOXC1-responsive reporter, and each region was individually capable of activating transcription when coupled to the GAL4-DB. These activation domains may not be entirely redundant. The C-terminal activation (AD-2) domain may serve as a general transcriptional activator capable of activating transcription from a number of promoters, because its fusion to the GAL4-DB created a potent activator that was more active then full-length FOXC1 fused to GAL4-DB. The N-terminal activation domain (AD-1) was more active in the context of FOXC1 than in GAL4 and therefore may be responsible for activation of FOXC1-specific target genes. AD-1 does not contain any motifs that are typically found in trans-activation domains (Fig. 6). It is not extensively rich in proline, glutamine, or acidic residues (25). This region does contain a poly-Ala repeat, and although these repeats are commonly found in repression domains (26), this element has been identified in the activation domains of other transcription factors (27). AD-2 contains a number of glutamine residues intermixed with bulky hydrophobic residues that are important for activation of transcription (25, 28). Natural FOXC1 promoters have yet to be identified. In this study we relied upon a synthetic FOXC1 reporter to map transcriptional regulatory domains. We are attempting to identify targets of FOXC1 trans-activation, and analysis of transcriptional regulatory domains will be monitored under more natural conditions.

Transcription activation domains have been identified for other members of the FOX family of proteins. Like FOXC1, both N- and C-terminal activation domains were required for full transcriptional activation by FOXA2 (20, 29). A single lung-specific C-terminal activation domain was identified in FOXF1, whereas two C-terminal activation domains were identified in FOXF2. Despite the high conservation of amino acid sequences in the FHD, there is little amino acid similarity in the activation domains between FOXC1 and other FOX proteins. However, the amino acid sequences of AD-1 and AD-2 are highly conserved among vertebrate FOXC1 homologues (Fig. 6), and this conservation suggests that these residues are critical for FOXC1 function. Furthermore, a frameshift mutation in the C-terminal region of FOXC1 has been identified in an AR patient (13). This mutation is predicted to delete the last 49 amino acids of FOXC1 and this protein would therefore lack key residues in the trans-activation domain. Because we have previously shown that FOXC1 point mutations that underlie the AR malformations result in reduced DNA binding and transcriptional activation of FOXC1 targets (19), this deletion of residues in the C-terminal activation domain may reduce FOXC1 trans-activation and produce a similar disease phenotype.

In addition to the identification of the FOXC1 activator domains, we provide evidence that FOXC1 possesses a transcriptional inhibitory domain located at amino acids 215–366 (Fig. 6). We propose that this region is an inhibitory domain for several reasons. First, removal of this region results in enhanced trans-activation by FOXC1. Second, transcriptional activation by FOXC1 AD-2 was higher than full-length FOXC1 when coupled to GAL4-DB, suggesting the presence of an inhibitory region in the full-length protein. Third, GAL4+AD2 fusions that contained these residues (GAL4+215–553) were less active than GAL4+AD2 proteins that did not contain this region (GAL4+366–553). Fourth, addition of residues 215–366 to the GAL4 AD impaired transcriptional activation. Together these observations suggest that residues 215–366 can reduce the activity of transcriptional activation domains. We refer to this region as an inhibitory domain (ID). This region may serve to modulate FOXC1 transcriptional activity. Although the FOXC1 ID contains a proline-rich segment similar to the WT-1 repressor protein (30), it was unable to repress transcription from the constitutively active SV40 promoter, suggesting the ID requires the presence of an AD to exert its negative effects. Like AD-1 and AD-2, the ID is conserved among vertebrate FOXC1 proteins (Fig. 6). A functionally similar ID domain exists in the osteoblast-specific transcription factor Osf2 (31). The 154 C-terminal residues of this protein inhibited an adjacent activation domain. Furthermore, like the FOXC1 ID, this inhibitory potential was transferable, because the Osf2 repressor domain was able to block transcription when fused to the transcriptional activator VP16 (31).

We provide evidence that FOXC1 is a phosphoprotein and that the activity of the inhibitory domain may be regulated by

![Fig. 6. Summary of FOXC1 functional domains.](http://www.jbc.org/content/journal/jbc/17/7/10296)
phosphorylation. An in silico prediction of the potential sites of phosphorylated residues in FOXC1 has revealed that 20 out of the 33 serine residues predicted to be phosphorylated lie in the FOXC1 ID (32). Phosphorylation of FOXC1 may limit the accessibility of accessory proteins necessary for full transcriptional activation. The FHD of FOXF2 interacts with TBP and TFIIB (24). This interacting region is conserved between FOXC1 and FOXF2 and suggests that a similar interaction may also occur between FOXC1 and TBP or TFIIB. The FOXC1 ID may recruit proteins that interfere with TBP and TFIIB binding to the nearby PHD. We are currently investigating the protein-protein interactions occurring with FOXC1 and if such interactions are regulated through phosphorylation.

Whether the enhanced activation observed by the removal of the FOXC1 inhibitory domain is related to the removal of phosphorylated residues in this region remains speculative at present. However, our observations predict that phosphorylation of this region may lead to an inhibition of FOXC1 function though the inhibitory domain. Negative regulation of FOX protein function by AKT phosphorylation has been observed for FOXO3 proteins (33). Regulation of FOXC1 by phosphorylation likely involves a different mechanism, because FOXC1 does not contain residues matching the AKT consensus site. Predicted phosphorylation sites that may phosphorylate FOXC1 include casein kinases I and II, glycerol synthase kinase 3, protein kinase A, and protein kinase C. Phosphorylation of transcription factors by these kinases can influence their transcriptional regulatory activity (34, 35). FOXC1 phosphorylation may occur at more than one residue based on the number of immunoreactive FOXC1 bands observed and the number of residues predicted to be phosphorylated (Fig. 6), which may prove problematic in FOXC1 activity (34, 35). FOXC1 phosphorylation may occur at more than one residue based on the number of immunoreactive FOXC1 bands observed and the number of residues predicted to be phosphorylated (Fig. 6), which may prove problematic in FOXC1 activity (34, 35). FOXC1 phosphorylation may occur at more than one residue based on the number of immunoreactive FOXC1 bands observed and the number of residues predicted to be phosphorylated (Fig. 6), which may prove problematic in FOXC1 activity (34, 35).

We have identified regions in the FOXC1 protein critical for its function as a transcription factor. The presence of FOXC1 IDAs along with an ID suggests that FOXC1 activity is under complex regulatory control with these multiple domains coordinating efficient transcriptional activation. Disease-causing point mutations in FOXC1 ultimately result in reduced transcriptional activation by FOXC1 (19). Chromosomal duplications involving FOXC1 were found in patients with ocular abnormalities (13, 36), suggesting that elevated levels or enhanced activity of FOXC1 may also be detrimental. Thus the precise control of FOXC1 activity is paramount for its proper function and the prevention of a disease phenotype. The identification of FOXC1 functional domains is an essential step in understanding how FOXC1 mutations may contribute to ocular diseases.

Acknowledgments—We thank M. Hughes for tissue culture services and Dr. A. Underhill for helpful discussions.

REFERENCES