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Transcriptional and DNA Binding Activity of the Foxp1/2/4 Family Is Modulated by Heterotypic and Homotypic Protein Interactions

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Foxp1, Foxp2, and Foxp4 are large multidomain transcriptional regulators belonging to the family of winged-helix DNA binding proteins known as the Fox family. Foxp1 and Foxp2 have been shown to act as transcriptional repressors, while regulatory activity of the recently identified Foxp4 has not been determined. Given the importance of this Fox gene subfamily in neural and lung development, we sought to elucidate the mechanisms by which Foxp1, Foxp2, and Foxp4 repress gene transcription. We show that like Foxp1 and Foxp2, Foxp4 represses transcription. Analysis of the N-terminal repression domain in Foxp1, Foxp2, and Foxp4 shows that this region contains two separate and distinct repression subdomains that are highly homologous termed subdomain 1 and subdomain 2. However, subdomain 2 is not functional in Foxp4. Screening for proteins that interact with subdomains 1 and 2 of Foxp2 using yeast two-hybrid analysis revealed that subdomain 2 binds to C-terminal binding protein 1, which can synergistically repress transcription with Foxp1 and Foxp2, but not Foxp4. Subdomain 1 contains a highly conserved leucine zipper similar to that found in N-myc and confers homo- and heterodimerization to the Foxp1/2/4 family members. These interactions are dependent on the conserved leucine zipper motif. Finally, we show that the integrity of this subdomain is essential for DNA binding, making Foxp1, Foxp2, and Foxp4 the first Fox proteins that require dimerization for DNA binding. These data reveal a complex regulatory mechanism underlying Foxp1, Foxp2, and Foxp4 activity, demonstrating that Foxp1, Foxp2, and Foxp4 are the first Fox proteins reported whose activity is regulated by homo- and heterodimerization.

Most transcriptional factors are modular proteins composed of DNA binding domains and domains and/or motifs that interact with other transcriptional regulators and modifying enzymes. Many of these interacting proteins do not bind to DNA directly but modulate DNA binding by conferring transcriptional activating or repressing activity to the DNA binding partner. This activity is often related to either compaction or relaxation of chromatin, thus restricting or permitting access of other transcriptional regulatory proteins. In this way, large multiprotein complexes assemble on enhancer and promoter sites, acting as complex switches that regulate gene expression.

Members of the Fox family of winged-helix transcription regulators are known to regulate cell fate and differentiation of various tissues (reviewed in reference 8). The Foxa subfamily of Fox genes has been shown to regulate various aspects of foregut endoderm development. Inactivation of Foxa2 results in severe morphological defects in foregut and floor plate development, leading to early embryonic death (2, 37). In the lung, both Foxa1 and Foxa2 are expressed early in development in the airway epithelium (11, 22). By late gestation, Foxa1 and Foxa2 expression becomes polarized along the proximal-distal axis of the developing epithelium, with the highest expression observed in the proximal airways. Foxa2 has been shown to regulate lung epithelium-specific gene expression, in particular the promoters for SP-A, SP-B, CC10, TTF-1/Nkx2.1, and Wnt7b (1, 5, 6, 17, 30, 31, 36). Overexpression of Foxa2 in the distal airways of the lung using the human SP-C promoter results in an arrest in lung epithelial development at the pseudoglandular stage and neonatal death (42). Remarkably, this ectopic expression inhibited surfactant protein gene expression, further supporting the notion that proper spatial and temporal expression of Foxa2 is required for lung development and maturation. Foxa2 also plays an important role in brain development. When overexpressed in the brains of transgenic mice, Foxa2 causes a reduction in the size of the cerebellum and reduced Pax-3 expression, while other putative targets of Foxa2, such as Foxa1 and bone morphogenetic protein 1, are ectopically expressed (29). Other Fox genes have also been shown to regulate diverse aspects of neural development. BF-1 (Foxg1) is required for normal proliferation and differentiation of telencephalic neuroepithelial cells, whereas a significant percentage of Fkh5$^{-/-}$ (Foxb1$^{-/-}$) embryos display an open neural tube and female Fkh5$^{-/-}$ mice that survive and reproduce have specific neural defects in the milk ejection reflex (19, 38).

In our effort to define the mechanisms underlying lung epithelial gene-specific transcription, we recently cloned three new Fox family members, Foxp1, Foxp2, and Foxp4, which are expressed in the lung, brain, and gut and act as repressors of lung-specific gene transcription (33). Our initial data showed that Foxp1 and Foxp2 are modular proteins consisting of a winged-helix DNA binding domain and a homologous DNA binding-dependent N-terminal transcriptional repression domain (33). The more recently described Foxp4 protein has not been analyzed to determine whether it is a transcriptional repressor, although its amino acid sequence is highly similar to

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that of Foxp1 and Foxp2 (21). All three genes are expressed at high levels in lung, neural, and gut tissues in distinct but overlapping patterns during embryogenesis and in the adult (21, 33). In addition, Foxp1 and Foxp2 were shown to repress gene transcription from the lung-specific CC10 promoter (33).

Recently, Foxp1 has been implicated as a tumor suppressor gene due to its loss in several types of tumors, including breast, lung, stomach, colon, and prostate tumors (3). The related Foxp3 gene has been shown to cause the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) in humans and the disease scurfy in mice (4, 7). Interestingly, Foxp2 has been implicated in the regulation of human speech through linkage in a specific familial language disorder, suggesting a role in neural development (20).

Given the importance of the Foxp1/2/4 family in regulating diverse developmental and disease processes, a thorough investigation into the mechanisms underlying their function is necessary to understand how these proteins regulate tissue-specific gene transcription. Foxp1, Foxp2, and Foxp4 contain a complex N-terminal region responsible for transcriptional repression (33). This region contains various protein-protein interaction motifs conserved in all three proteins, including a zinc finger and a putative leucine zipper. However, the importance of each of these domains and motifs has remained uncharacterized.

To further define the mechanisms underlying the function of Foxp1, Foxp2, and Foxp4, we have delineated the domains and protein-protein interactions required for transcriptional repression and DNA binding. We show that there are two separate and distinct transcriptional repression subdomains (subdomains 1 and 2) in Foxp1 and Foxp2 but only subdomain 1 is functionally present in Foxp4. We show that subdomain 2 mediates interactions with the corepressor protein C-terminal binding protein 1 (CtBP-1) in both yeast two-hybrid and coimmunoprecipitation analysis. We further show that CtBP-1 synergistically represses transcription through Foxp1 and Foxp2 but not through Foxp4, in agreement with the presence or absence of a CtBP-1 binding motif. Importantly, we present data demonstrating that the leucine zipper motif is responsible for hetero- and homotypic interactions among the Foxp1, Foxp2, and Foxp4 proteins and mutations in this region found in humans eliminate these interactions. Moreover, the integrity of the leucine zipper motif is required for DNA binding of Foxp1, Foxp2, and Foxp4 and thus transcriptional activity. Taken together, these data demonstrate that Foxp1, Foxp2, and Foxp4 require dimerization to bind DNA and repress transcription, which is a novel feature of these Fox proteins. Along with their ability to interact with corepressor molecules, such as CtBP-1, these data support the idea that Foxp1, Foxp2, and Foxp4 are at the core of a multiprotein complex that regulates diverse aspects of tissue-restricted gene expression during development in a way distinct from other Fox proteins.

MATERIALS AND METHODS

Generation of plasmids, cotransfection assays, and reverse transcriptase PCR (RT-PCR). The full-length Foxp1 and Foxp2 expression plasmids have been described previously (33). The full-length Foxp4 expression plasmid was generated by PCR amplification of the entire open reading frame of the mouse Foxp4 cDNA and subcloning it into the pCMVTag3B expression vector which contains the c-myc epitope tag at the N terminus (Stratagene). To generate the pCMV.luc reporter, nucleotides 454 to 888 containing a portion of the cytomegalovirus (CMV) promoter were amplified from the pcDNA3 plasmid and cloned into the pGL3 basic luciferase (luc) reporter plasmid (Promega). This region of the CMV promoter lacked a consensus 5′-TRTTKARY-3′ Fox DNA binding site but retained high levels of activity in cell culture. The nucleotide sequences corresponding to the indicated amino acid sequences for Foxp1, Foxp2, and Foxp4 were amplified by PCR and subcloned into the *Eco*RI and *Xba*I sites of the pGAL4 vector to generate expression plasmids for GAL4 DNA binding domain fusion proteins (23). In-frame deletions and mutations were generated by PCR specifically to delete either the entire leucine zipper motif (Foxp1 amino acids [aa] 373 to 400, Foxp2 aa 384 to 410, or Foxp4 aa 352 to 376) or the indicated glutamic acid residues in Foxp1, Foxp2, and Foxp4. For detection of transcriptional repression using the pGAL4 fusion proteins, NIH 3T3 cells were transfected with 0.5 μg of pGAL4SV40.luc reporter vector and 2.5 μg of the indicated pGAL4 fusion constructs along with 0.5 μg of pMSVβgal control vector. The pGAL4SV40.luc reporter plasmid contains four GAL4 DNA binding sites upstream of the simian virus 40 (SV40) promoter (34). For reporter assays in H441 cells, 0.5 μg of pCC10.luc reporter plasmid (33), 2.5 μg of Foxp1, Foxp2, or Foxp4 expression plasmid, and 0.5 μg of pMSVβgal plasmid were transfected into H441 cells using Fugene 6 as previously described (33). For the CtBP-1 cotransfection assays, Foxp1, Foxp2, and Foxp4 expression plasmids (1 μg) were transfected along with the indicated amounts of pcDNA3.1/CtBP-1 expression plasmid (39) to detect the level of synergistic repression. The amino acid sequence of the CtBP-1 binding sites in Foxp1 and Foxp2 were changed from PLNLV to AANAA using PCR mutagenesis and cloned into the pCMVTag3B vector. Luciferase and β-galactosidase activity were measured using commercially available kits (Promega, Madison, Wis.). Data are shown as the averages ± standard errors of the means of three assays.

RT-PCR was performed using Superscript II RT and total RNA from H441 cells. Oligonucleotides specific for CtBP-1 and CtBP-2 were used to amplify cDNA-specific fragments of each transcript as indicated.

Yeast two-hybrid assays. The region of the mouse Foxp2 cDNA encoding aa 260 to 500 was cloned into the pGBKT7 bait plasmid in frame with the GAL4 DNA binding domain, which was used to screen an adult rat lung yeast two-hybrid library according to the manufacturer's directions (Clontech, Inc.). Library plasmids from yeast colonies that grew on quadruple dropout medium (without tryptophan, leucine, histidine, and adenine) and turned blue in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-α-Gal) (which measures activation of the *MEL1* reporter gene) were isolated from yeast and transformed into *Escherichia coli*. All positive clones were retested in the yeast two-hybrid assay by retransfection and replating on selection medium (SD without tryptophan, leucine, histidine, and adenine but with X-α-gal). Approximately 10^6 primary clones were screened, which resulted in 24 positive clones that were repeatedly positive in the yeast two-hybrid assay. The identity of isolated clones was determined by DNA sequence analysis.

Coimmunoprecipitation assays. Coimmunoprecipitation assays were performed by transfecting HEK-293 cells with the indicated Foxp1, Foxp2, and Foxp4 FLAG-tagged or c-myc-tagged wild-type or mutant cDNAs expressed from the pCMVTag vectors (Stratagene) as indicated using Fugene 6 (Roche Biochemicals). For CtBP-1 coimmunoprecipitation assays, Foxp1, Foxp2, and Foxp4 plasmids were cotransfected with a c-myc-tagged CtBP-1 expression vector into HEK-293 cells. After 48 h, cells were harvested and nuclear extracts were prepared as described previously (36) and diluted in immunoprecipitation buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 0.5 mM EDTA, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor cocktail [Sigma]). After insoluble cellular material was removed by centrifugation and filtration, half of the diluted nuclear extract was subjected to immunoprecipitation with either the anti-myc monoclonal antibody (9E10) or anti-FLAG (M2) antibody as indicated for 2 h at 4°C; the antibody was coupled to protein A/G Sepharose (Santa Cruz Biotechnology). A portion of the nuclear extracts were used for immunoblotting to detect expression levels of the indicated proteins. The immunoprecipitates were washed three times with immunoprecipitation buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with either the anti-c-myc monoclonal antibody or anti-FLAG antibody as indicated.

EMSA. A glutathione-*S*-transferase (GST)-Foxp2 fusion protein was generated by cloning the cDNA sequence encoding aa 457 to 714 of the mouse Foxp2 protein into the pGEX4T1 plasmid. GST-Foxp2 and GST proteins were expressed and purified from *E. coli* as previously described (24). For cleavage of the GST-Foxp2 fusion protein, 5 μg of GST-Foxp2 and 10 U of thrombin were incubated at room temperature for 18 h before analysis by SDS-PAGE and electrophoretic mobility shift assays (EMSA). In vitro-translated proteins were generated using the TNT reticulocyte lysate system (Promega). EMSA were

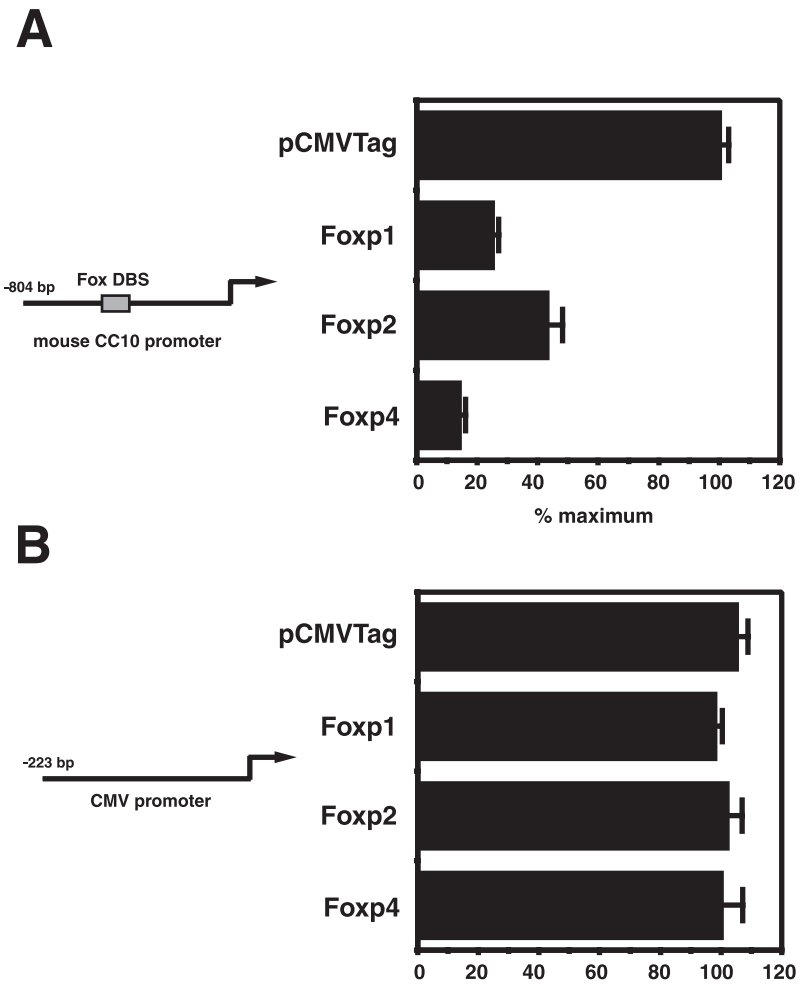

FIG. 1. Foxp4 represses lung-specific gene transcription. H441 cells were cotransfected with pCMVTag3B (pCMVTag), pCMV/Foxp1 (Foxp1), pCMV/Foxp2 (Foxp2), or pCMV/Foxp4 (Foxp4) expression plasmid and with the pCC10.luc (A) or pCMV.luc (B) reporter plasmid along with the pMSVβgal reference plasmid. Forty-eight hours after transfection, cells were harvested, and luciferase activity was measured and normalized to the activity obtained after transfection with the pCMVTag3B plasmid. All assays included the pMSVβgal control plasmid, and differences in transfection efficiencies were corrected using a commercial β-galactosidase assay. Assays were performed in triplicate, and the data are presented as the maximum percentage of relative luciferase activity obtained upon cotransfection of either reporter plasmid with the pCMVTag3B plasmid ± standard error of the mean. DBS, DNA binding site.

performed as previously described using the mouse CC10 Fox DNA binding oligonucleotide (5′-TGAAAAGAGATTATTTGCTTATTCCACGGAGAAGA TGACAAGTAAATAATG-3′) and either 5 μl of in vitro-translated Foxp1, Foxp2, and Foxp4 or 500 ng of GST or GST-Foxp2 proteins (36). For supershift EMSA, 1 μl of Foxp1, Foxp2, or Foxp4 rabbit antiserum was preincubated with the extracts for 30 min prior to the EMSA.

RESULTS

Characterization of Foxp4 as a transcriptional repressor. To determine whether Foxp4 could repress lung-specific gene expression, expression vectors containing the full-length Foxp1, Foxp2, and Foxp4 cDNAs were cotransfected with the pCC10.luc reporter into H441 lung epithelial cells. The mouse CC10 promoter contains a consensus Fox protein DNA binding sequence, and the CC10 promoter is active in H441 cells (33). Upon cotransfection with Foxp1, Foxp2, or Foxp4 plasmids, activity of the mouse CC10 promoter was significantly repressed (Fig. 1A). Since mutation of the Fox DNA binding site in the CC10 promoter dramatically reduces the expression of linked cDNAs in H441 cells (30) (data not shown), we cotransfected Foxp1, Foxp2, and Foxp4 plasmids along with a CMV promoter that lacked a Fox DNA binding site to determine the specificity of this repression. This CMV promoter was unaffected by coexpression of Foxp1, Foxp2, or Foxp4 (Fig. 1B). Together, these data show that like Foxp1 and Foxp2, Foxp4 represses lung-specific gene transcription.

Identification of two distinct repression domains in Foxp1, Foxp2, and Foxp4. We had previously shown that the N terminus of Foxp1 and Foxp2 conferred transcriptional repression when fused to the GAL4 heterologous DNA binding domain (33). Of note, this region is highly conserved among Foxp1, Foxp2, and Foxp4 proteins, although the specific domains within this region required for repression have remained uncharacterized. To determine the minimal sequences required for transcriptional repression by Foxp1, Foxp2, and Foxp4, GAL4 fusion proteins that included distinct regions of Foxp2 from within the previously defined N-terminal repres-

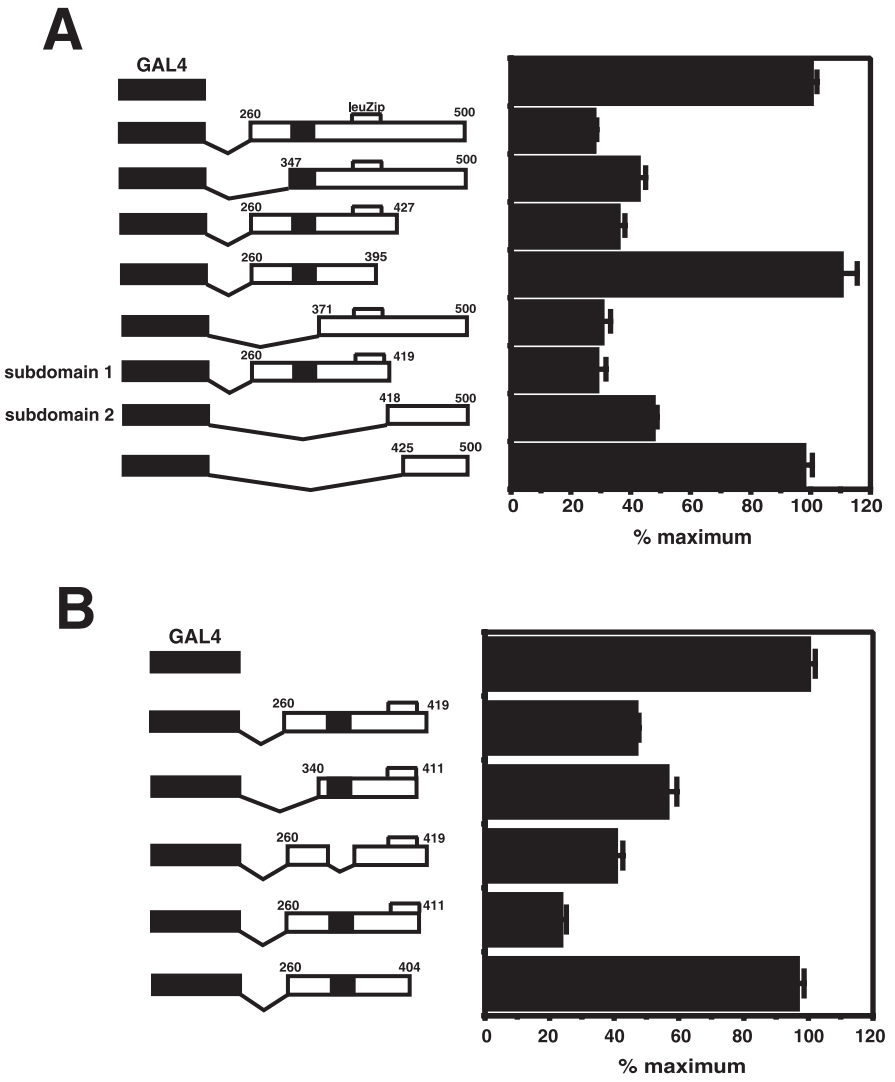

FIG. 2. Identification of subdomains 1 and 2 in Foxp2 protein. NIH 3T3 cells were transfected with the pGAL4SV40.luc reporter plasmid along with expression plasmids encoding the GAL4 DNA binding domain or GAL4 chimeric expression vectors encoding the indicated regions of the mouse Foxp2 protein. Forty-eight hours after transfection, cells were harvested, and luciferase activity was measured and normalized to the activity obtained after transfection with the pGAL4 plasmid. (A) Definition of subdomains 1 and 2 as aa 260 to 419 and aa 418 to 500, respectively. (B) The zinc finger of subdomain 1 is not essential for transcriptional repression. All assays included the pMSVβgal control plasmid, and differences in transfection efficiencies were corrected using a commercial β-galactosidase assay. Assays were performed in triplicate, and the data are presented as the maximum percentage of relative luciferase activity obtained upon cotransfection of the pGAL4SV40.luc reporter plasmid with the pGAL4 plasmid ± standard error of the mean. GAL4 fusion proteins delineating subdomains 1 and 2 are indicated on the left in panel A. The zinc finger (black box) and leucine zipper (leuZip) (bracket) motifs are indicated. All GAL4 fusion proteins were expressed at approximately equal levels (data not shown).

sion domain were generated. These regions were designed to contain or eliminate certain protein motifs, such as the zinc finger and putative leucine zipper previously described (33). The largest segment of Foxp2 examined, aa 260 to 500, significantly repressed transcription (Fig. 2A). Truncation of sequences from aa 260 to 346, 428 to 500, or 420 to 500 did not affect repression (Fig. 2A). Further mapping of this region of Foxp2 revealed that sequences between aa 404 to 411 are required for transcriptional repression (Fig. 2B). Moreover, deletion of the conserved zinc finger motif did not appreciably affect repression in this domain of Foxp2 (Fig. 2B). Two distinct domains, subdomain 1 (aa 260 to 419) and subdomain 2 (aa 418 to 500), both showed significant levels of transcriptional repression, suggesting that there are at least two different domains within Foxp2 responsible for transcriptional repression.

Regions within Foxp1 and Foxp4 correlating to subdomains 1 and 2 were also tested for transcriptional repression. As shown in Fig. 3A, subdomain 1 is conserved in both Foxp1 and Foxp4 (Fig. 3A). In contrast, subdomain 2 is conserved in Foxp1, while the same region in Foxp4 did not confer repression to the GAL4 DNA binding domain (Fig. 3B). Full-length Foxp1/2/4 repressed transcription when fused to the GAL4 DNA binding domain (Fig. 3C). These data pointed to conservation of subdomain 1 but significant differences in subdomain 2 in Foxp1, Foxp2, and Foxp4.

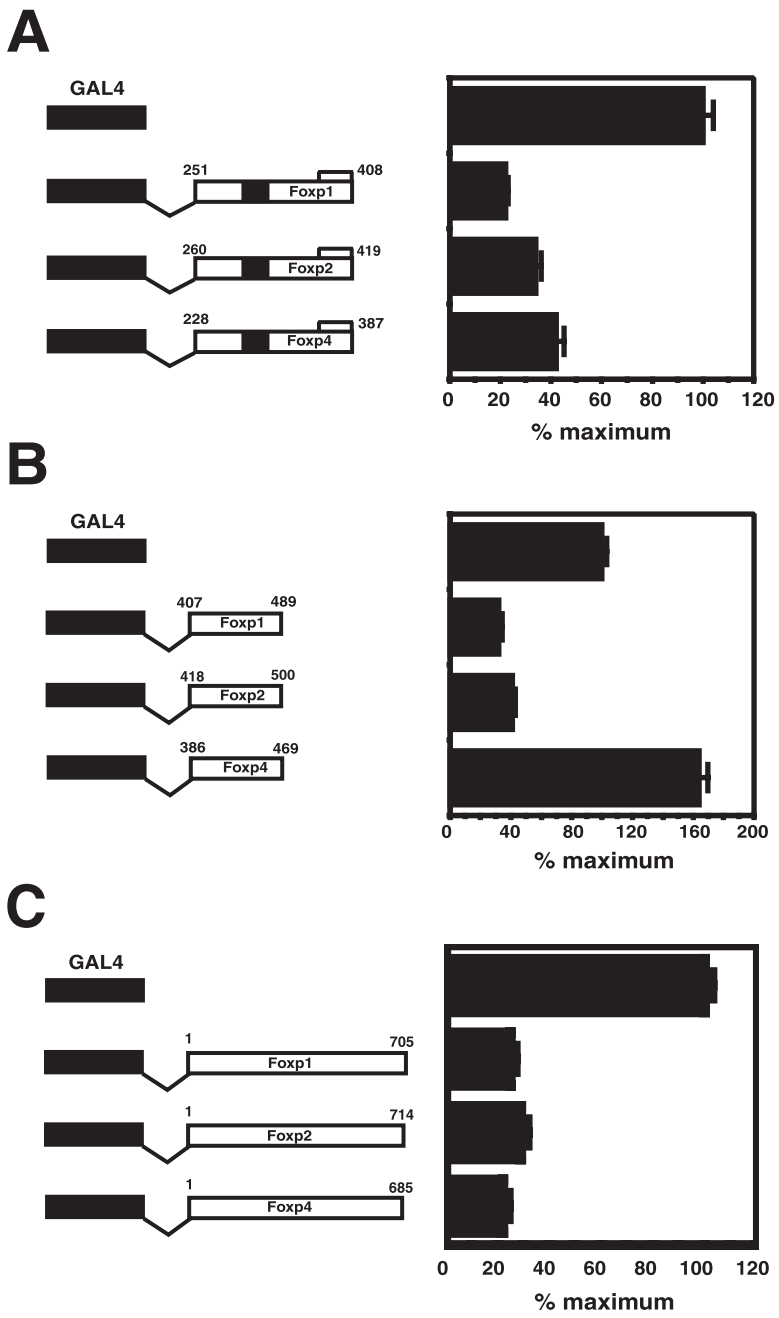

FIG. 3. Conservation of subdomain 1 but not subdomain 2 in Foxp1, Foxp2, and Foxp4 proteins. NIH 3T3 cells were transfected with the pGAL4SV40.luc reporter plasmid along with expression plasmids encoding the GAL4 DNA binding domain or GAL4 chimeric expression vectors encoding the indicated regions of the mouse Foxp1, Foxp2, and Foxp4 proteins. Forty-eight hours after transfection, cells were harvested, and luciferase activity was measured and normalized to the activity obtained after transfection with the pGAL4 plasmid. All assays included the pMSVβgal control plasmid, and differences in transfection efficiencies were corrected using a commercial β-galactosidase assay. Assays were performed in triplicate, and the data are presented as the maximum percentage of relative luciferase activity obtained upon cotransfection of the pGAL4SV40.luc reporter plasmid with the pGAL4 plasmid ± standard error of the mean. Note the conservation of repression in subdomain 1 (A) but not subdomain 2 (B) in Foxp1, Foxp2, and Foxp4. However, full-length Foxp1, Foxp2, and Foxp4 proteins display similar repression activity when fused to GAL4 (C). The zinc finger motif is indicated by a black box in panel A. All GAL4 fusion proteins were expressed at approximately equal levels (data not shown).

Corepressor CtBP-1 binds and represses transcription through Foxp1 and Foxp2. To identify putative interacting partners for Foxp1, Foxp2, and Foxp4 which modulate their function, an adult rat lung yeast two-hybrid library was screened using aa 260 to 500 of Foxp2 as the bait. This screen yielded 24 clones which were able to grow on the minimal dropout medium and activated the *MEL1* gene which provided a positive color reaction when X-α-Gal was included in the medium (data not shown). Sequencing of these clones revealed that 14 were found to encode the rat orthologue of CtBP-1.

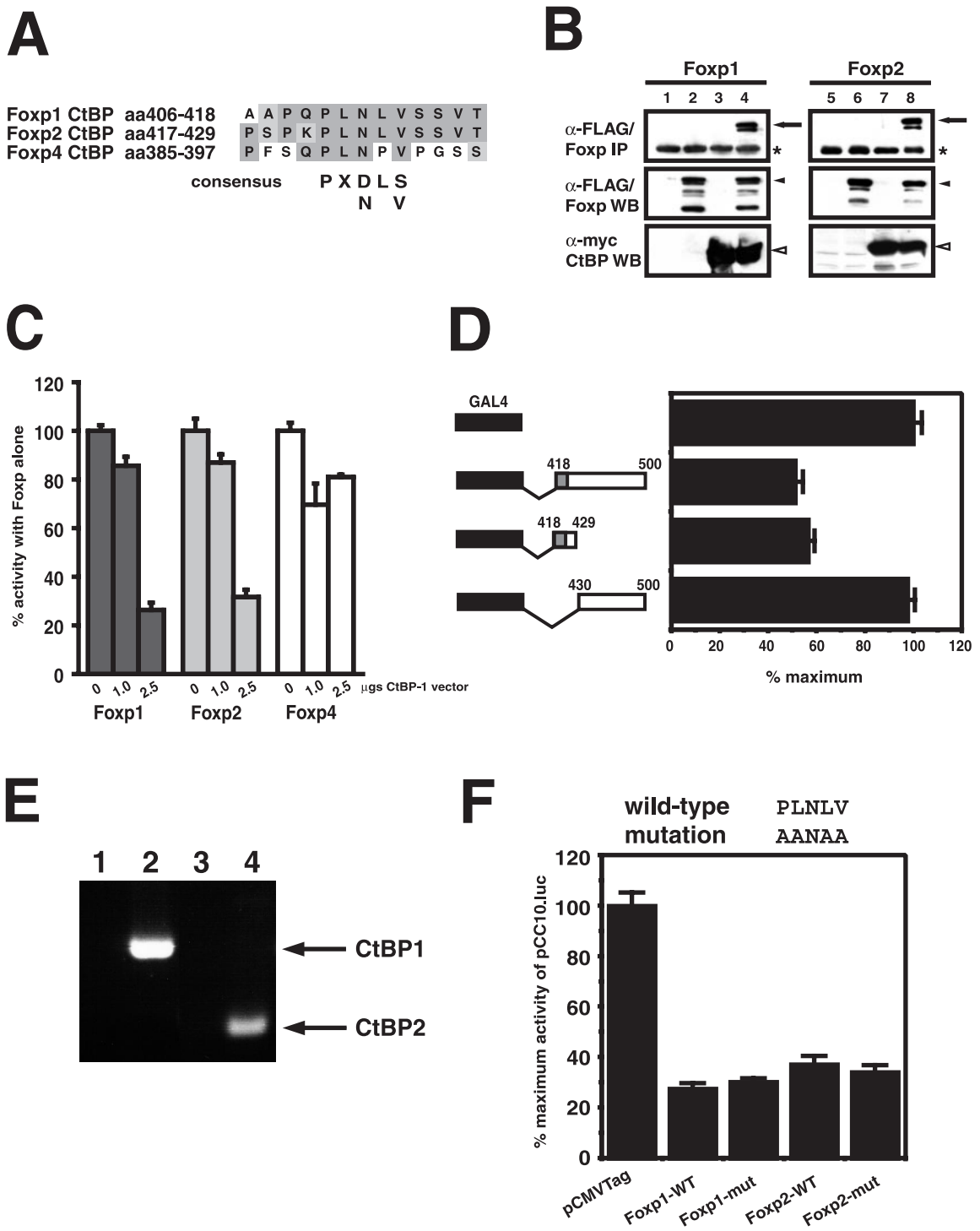

FIG. 4. The corepressor CtBP-1 interacts with and represses transcription through Foxp1 and Foxp2 but not Foxp4. (A) The CtBP-1 (CtBP) binding motif is conserved in Foxp1 and Foxp2 but not Foxp4. Note the change from a conserved leucine to a proline. (B) CtBP-1 coimmunoprecipitates with Foxp1 and Foxp2. HEK-293 cells were cotransfected with either pCMV/Foxp1 (lanes 2 and 4), pCMV/Foxp2 (lanes 6 and 7), and pCMV/CtBP-1 (lanes 3, 4, 7, and 8) or with pCMVTag3B control vector (lanes 1 and 5). The Foxp1 and Foxp2 proteins were FLAG tagged, and CtBP-1 was myc tagged. Forty-eight hours after transfection, coimmunoprecipitations were performed on cell extracts using an anti-c-myc (α-myc) monoclonal antibody (9E10). Immunoprecipitates (IP) were probed with an anti-FLAG (α-FLAG) monoclonal antibody (M2). The presence of Foxp1 and Foxp2 coimmunoprecipitating with CtBP-1 is indicated by arrows. The cross-reactivity of the secondary antibody to the heavy chain of immunoglobulin G is indicated by asterisks. Expression of Foxp1 or Foxp2 (black arrowheads) or CtBP-1 (white arrowheads) in Western blots (WB) of cell extracts is indicated. (C) H441 cells were transfected with the pCC10.luc reporter plasmid along with expression plasmids encoding the full-length mouse Foxp1, Foxp2, and Foxp4 proteins and increasing amounts of the pCMV/CtBP-1 expression plasmid. Forty-eight hours after transfection, cells were harvested, and luciferase activity was measured and normalized to the activity obtained after transfection with the pCC10.luc plasmid and the pCMV/Foxp1, pCMV/Foxp2, and pCMV/Foxp4 plasmids without the pCMV/CtBP-1 expression plasmid. (D) Trun-

CtBP-1 has been shown to act as a corepressor for several transcription factors including the E1A viral protein, FOG-1/2, ZEB, and class II histone deacetylases (12, 13, 15, 27, 32, 39). A putative CtBP-1 binding site was found in Foxp1 and Foxp2 in the region delineated as subdomain 2 (Fig. 4A). However, this site is significantly altered in Foxp4, with a proline substituted at position 4 where a conserved and essential leucine is found, suggesting that CtBP-1 may not functionally interact with Foxp4 (Fig. 4A). To determine whether CtBP-1 was able to bind to Foxp1 and Foxp2 in vivo, cotransfection experiments were performed in HEK-293 cells with FLAG-tagged Foxp1 and Foxp2 and c-myc-tagged CtBP-1. Both Foxp1 and Foxp2 were immunoprecipitated in a complex with CtBP-1, confirming the protein-protein interaction (Fig. 4B). However, CtBP-1 did not bind to Foxp4 (data not shown). To test whether CtBP-1 could affect repression through Foxp1, Foxp2, and Foxp4, cotransfection experiments were performed and the amount of CtBP-1 expression plasmid was titrated. Increasing amounts of CtBP-1 lead to a dose-dependent increase of repression through Foxp1 and Foxp2 but not Foxp4, which correlates with the presence of a CtBP-1 site in Foxp1 and Foxp2 but not in Foxp4 (Fig. 4C). These results show that CtBP-1 functionally interacts with Foxp1 and Foxp2 but not Foxp4.

To determine whether the CtBP-1 binding site was necessary for transcriptional repression mediated by subdomain 2, GAL4 fusion proteins were generated from the Foxp2 sequence either containing or lacking this site. As shown in Fig. 4D, truncation of the CtBP-1 site resulted in the loss of repression by subdomain 2. Moreover, the presence of aa 418 to 429, which contains the CtBP-1 binding site, was able to confer transcriptional repression, suggesting that this motif is sufficient to confer transcriptional repression to the GAL4 DNA binding domain (Fig. 4D). Of note, both CtBP-1 and CtBP-2 are expressed in H441 cells (Fig. 4E).

To determine whether the CtBP-1 site was essential for repression mediated by the full-length Foxp1 and Foxp2 proteins, this site was mutated in each full-length protein, and cotransfection assays were performed using the pCC10.luc reporter plasmid in H441 cells. Mutation of the CtBP-1 site in both Foxp1 and Foxp2 did not significantly alter their ability to repress transcription, suggesting that CtBP-1 may be important but is not essential for transcriptional repression mediated by Foxp1 and Foxp2 (Fig. 4F). Along with the absence of a functional CtBP-1 binding site in Foxp4, these data suggest that there are additional mechanisms underlying transcriptional repression mediated by Foxp1, Foxp2, and Foxp4.

The leucine zipper is important for transcriptional repression mediated by subdomain 1 in Foxp1, Foxp2, and Foxp4. We next tested whether the leucine zipper motif located in subdomain 1 was important for transcriptional repression by Foxp1, Foxp2, and Foxp4. This motif is similar to that found in the N-myc protein and is located within a conserved α-helix in Foxp1, Foxp2, Foxp3, and Foxp4 (Fig. 5A). To test whether this leucine zipper motif was important for transcriptional repression mediated by subdomain 1 of the Foxp1, Foxp2, and Foxp4 proteins, point mutations were introduced into each of these GAL4 fusion proteins to change the fourth conserved leucine position to an alanine (Fig. 5B and C). Cotransfection assays show that these mutations significantly reduced transcriptional repression by subdomain 1 of Foxp1, Foxp2, and Foxp4 (Fig. 5B and C). Together, these data suggest that this leucine zipper motif is important for transcriptional repression mediated by the Foxp1, Foxp2, and Foxp4 proteins.

Foxp1, Foxp2, and Foxp4 can homo- and heterodimerize through the conserved leucine zipper motif. Leucine zipper motifs are responsible for homo- and heterodimerization of several proteins. Although Fox proteins have been demonstrated to bind to DNA as monomers and there have been no reports of Fox proteins capable of homo- or heterodimerizing (10), the presence of a leucine zipper in Foxp1, Foxp2, and Foxp4 suggested that these proteins may homo- and/or heterodimerize with each other. To determine whether Foxp1, Foxp2, and Foxp4 are capable of dimerization, coimmunoprecipitation analysis was performed using nuclear extracts from HEK-293 cells transfected with various combinations of Foxp1, Foxp2, and Foxp4 expression vectors. All three proteins were found to homodimerize with themselves and to heterodimerize with each other (Fig. 6A). To determine whether the leucine zipper was essential for these interactions, in-frame deletions of the leucine zipper domains were generated for Foxp1, Foxp2, and Foxp4 (Δleuzip) and used again in coimmunoprecipitation assays. Deletion of the leucine zipper motif eliminated homo- and heterodimerization of Foxp1, Foxp2, and Foxp4 (Fig. 6C). All proteins were expressed at equivalent levels (Fig. 6B and D). The results of these assays show that the leucine zipper is essential for Foxp1, Foxp2, and Foxp4 dimerization.

Recently, a mutation in the related Foxp3 gene in humans was discovered. This mutation resulted in the in-frame deletion of E251, which is located in the conserved leucine zipper (Fig. 7A) (9). Patients with this mutation exhibit the same phenotype as patients containing mutations in the forkhead DNA

cation of the CtBP-1 site in subdomain 2 of Foxp2 eliminates transcriptional repression by this domain and demonstrates that aa 418 to 429 are sufficient for repression by this domain. NIH 3T3 cells were transfected with expression vectors encoding the indicated Foxp2-GAL4 fusion proteins. Luciferase assays were performed, and luciferase activity was normalized as described in the legends to Fig. 1 to 3. All GAL4 fusion proteins were expressed at approximately equal levels (data not shown). (E) RT-PCR using H441 cell cDNA to detect CtBP-1 and CtBP-2 expression. Lanes 1 and 2 used oligonucleotides specific for CtBP-1, while lanes 2 and 3 used oligonucleotides specific for CtBP-2. Reaction mixtures lacking RT (lanes 1 and 3) and reaction mixtures containing RT (lanes 2 and 4) were used. (F) Mutation of the CtBP-1 binding site in Foxp1 and Foxp2 does not affect repression of the mouse CC10 promoter. Mutations were introduced into the CtBP-1 binding motif in Foxp1 and Foxp2 (Materials and Methods), and these plasmids (Foxp1-mut and Foxp2-mut) were transfected along with wild-type pCMV/Foxp1 (Foxp1-WT) and pCMV/Foxp2 (Foxp2-WT) plasmids and the pCC10.luc reporter plasmid into H441 cells. Forty-eight hours after transfection, cells were harvested, and luciferase activity was measured and normalized to the activity obtained after transfection with the pCC10.luc plasmid and the pCMVTag3B expression plasmid. All assays included the pMSVβgal control plasmid, and differences in transfection efficiencies were corrected using a commercial β-galactosidase assay. Assays were performed in triplicate, and the data are presented as the maximum percentage of relative luciferase activity obtained upon cotransfection of the pGAL4SV40.luc reporter plasmid with the pGAL4 plasmid ± standard error of the mean.

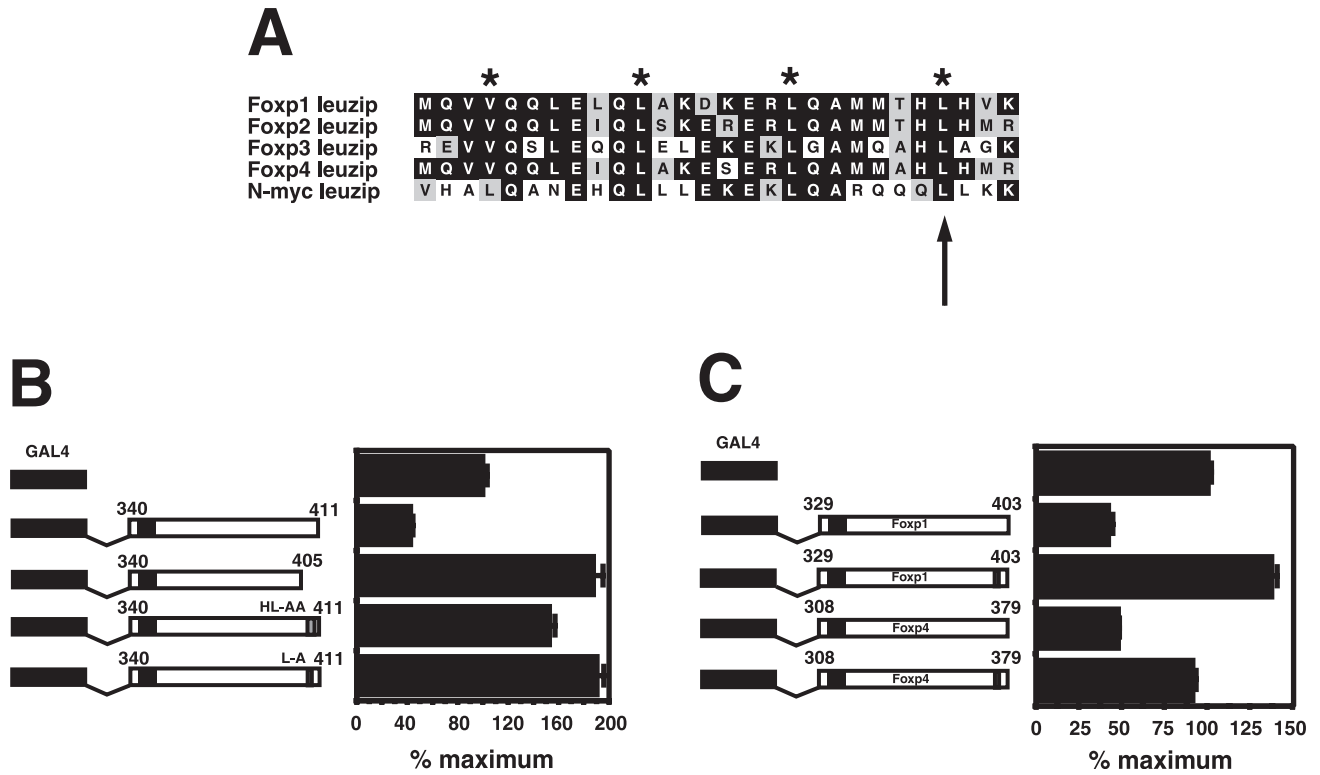

FIG. 5. The leucine zipper motif is important for repression by subdomain 1. (A) Alignment of the leucine zipper (leuzip) motifs of Foxp1, Foxp2, Foxp3, Foxp4, and N-myc, showing the conservation of this region. Identical amino acids (white letters on black background) and similar amino acids (light gray shaded background) are indicated. Asterisks denote conserved positions of leucines, or in the case of Foxp1, Foxp2, Foxp3, and Foxp4, a valine substitution at the first position. The leucine mutated in these experiments is denoted with an arrow. (B) Mutation of the fourth leucine in Foxp2 eliminates repression of subdomain 1. NIH 3T3 cells were transfected with the pGAL4SV40.luc reporter plasmid along with expression plasmids encoding the GAL4 DNA binding domain or GAL4 chimeric expression vectors encoding the indicated regions of the Foxp2 protein. Forty-eight hours after transfection, cells were harvested, and luciferase activity was measured and normalized to the activity obtained after transfection with the pGAL4 plasmid. Note that mutation of either aa 407 and 408 (HL to AA) or just the leucine at aa 408 to alanine (L to A) results in a loss of repression by subdomain 1. (C) Leucine zipper mutations in subdomain 1 of Foxp1 and Foxp4 also eliminate repression. NIH 3T3 cells were transfected with the pGAL4SV40.luc reporter plasmid along with expression plasmids encoding the GAL4 DNA binding domain or GAL4 chimeric expression vectors encoding the indicated regions of the Foxp1 and Foxp4 proteins. Forty-eight hours after transfection, cells were harvested, and luciferase activity was measured and normalized to the activity obtained after transfection with the pGAL4 plasmid. Note loss of repression by mutation of the fourth conserved leucine in subdomain 1 of Foxp1 (aa 397) and Foxp4 (aa 376). All assays included the pMSVβgal control plasmid, and differences in transfection efficiencies were corrected using a commercial β-galactosidase assay. Assays were performed in triplicate, and the data are presented as the maximum percentage of relative luciferase activity obtained upon cotransfection of the pGAL4SV40.luc reporter plasmid with the pGAL4 plasmid ± standard error of the mean. All GAL4 fusion proteins were expressed at approximately equal levels (data not shown).

binding domain. This mutation is predicted to disrupt the spacing of the conserved leucines, thus potentially disrupting the leucine zipper motif, supporting the hypothesis that this conserved leucine zipper is essential for function of Foxp1, Foxp2, Forp3, and Foxp4 proteins. To determine the effect this mutation would have on dimerization, in-frame deletions of E388, E399, and E367 in Foxp1, Foxp2, and Foxp4, respectively, were generated and used in coimmunoprecipitation assays (ΔE mutations). These mutations eliminated the ability of Foxp1, Foxp2, and Foxp4 to dimerize with themselves and each other, demonstrating that dimerization requires an intact leucine zipper motif (Fig. 7B).

Dimerization is required for transcriptional activity and DNA binding by Foxp1, Foxp2, and Foxp4. Since mutations that delete the conserved E251 in Foxp3 appear to result in a complete loss of function and, as shown above, deletion of either the entire leucine zipper or the conserved glutamic acid results in a loss of dimerization of Foxp1, Foxp2, and Foxp4 proteins, we sought to determine what effect these mutations would have on the transcriptional activity and DNA binding of Foxp1, Foxp2, and Foxp4. Cotransfection assays were performed with the expression constructs described above encoding the Δleuzip and ΔE mutations in Foxp1, Foxp2, and Foxp4 along with the pCC10.luc reporter plasmid in H441 cells. Both Δleuzip and ΔE mutations dramatically reduce the ability of Foxp1, Foxp2, and Foxp4 to repress transcription (Fig. 8).

EMSA were performed to determine the effect of the Foxp1, Foxp2, and Foxp4 ΔE mutations on DNA binding to the consensus forkhead site in the CC10 promoter. As we had previously determined, wild-type Foxp1, Foxp2, and Foxp4 bind efficiently to this site (Fig. 9A). Surprisingly, little to no binding was observed with the ΔE mutations, even though equal amounts of protein were used in the assay (Fig. 9A and B). These data suggested that disruption of the leucine zipper

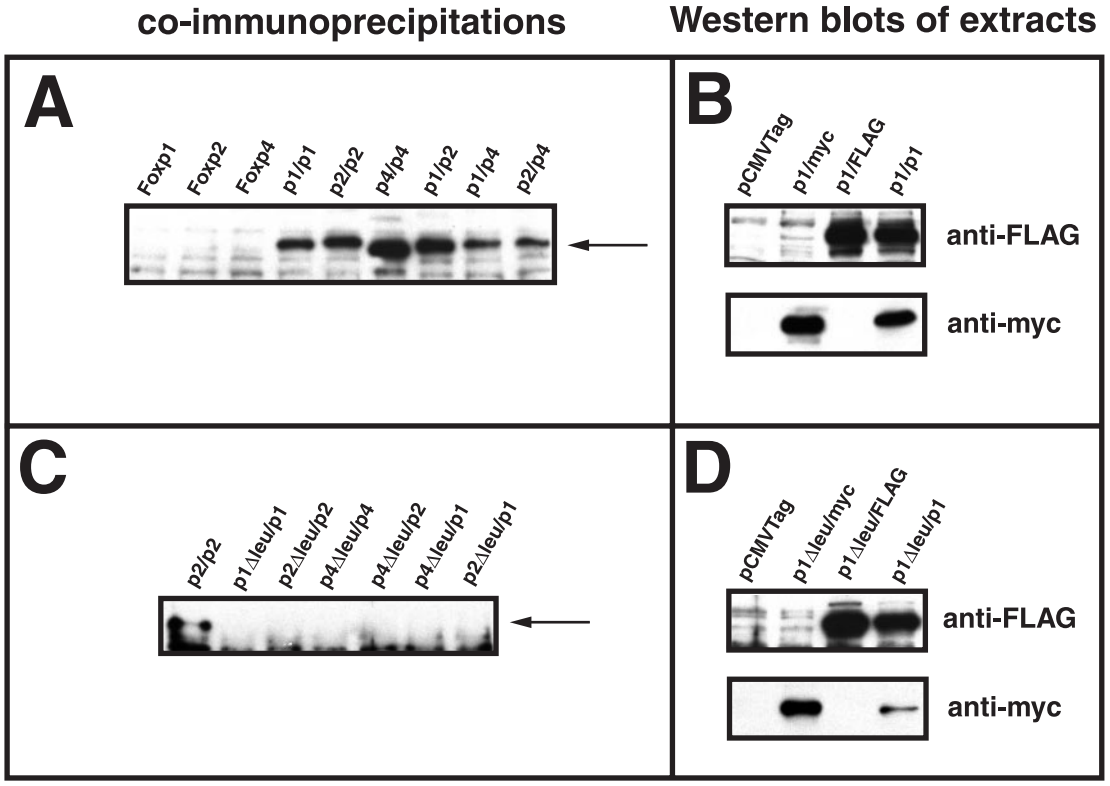

FIG. 6. The leucine zipper motif in Foxp1, Foxp2, and Foxp4 mediates homo- and heterodimerization. HEK-293 cells were transfected with combinations of expression vectors encoding Foxp1, Foxp2, and Foxp4 proteins that had been tagged with FLAG or c-myc. The c-myc monoclonal antibody (9E10) was used to immunoprecipitate proteins from cell extracts. Immunoprecipitated proteins were resolved on SDS-polyacrylamide gels or Western blots, which were probed with the anti-FLAG M2 monoclonal antibody to reveal coimmunoprecipitated proteins. The first protein listed above each lane was tagged with c-myc, while the second protein was tagged with FLAG. The cells were transfected by plasmids encoding myc-tagged Foxp1 (Foxp1), myc-tagged Foxp2 (Foxp2), myc-tagged Foxp4 (Foxp4), myc-tagged Foxp1 and FLAG-tagged Foxp1 (p1/p1), myc-tagged Foxp2 and FLAG-tagged Foxp2 (p2/p2), myc-tagged Foxp4 and FLAG-tagged Foxp4 (p4/p4), myc-tagged Foxp1 and FLAG-tagged Foxp2 (p1/p2), myc-tagged Foxp1 and FLAG-tagged Foxp4 (p1/p4), myc-tagged Foxp2 and FLAG-tagged Foxp4 (p2/p4) proteins. (B) Western blots of sample cell lysate showing expression of myc- and FLAG-tagged Foxp1. Lysates from pCMVTag3B-transfected (pCMVTag), myc-tagged Foxp1 plasmid-transfected (p1/myc), FLAG-tagged Foxp1 plasmid-transfected (p1/FLAG), and both myc- and FLAG-tagged Foxp1 plasmid-transfected (p1/p1) HEK-293 cells were used. (C) Coimmunoprecipitation assays using Foxp1, Foxp2, and Foxp4 leucine zipper deletion (Δleu) mutants. Results are configured as in panel A, with the first protein being myc tagged and the second protein being FLAG tagged. The c-myc monoclonal antibody was used for immunoprecipitations, while the anti-FLAG monoclonal antibody was used on Western blots for immunodetection of coimmunoprecipitated proteins. The cells were transfected with myc-tagged Foxp2 and FLAG-tagged Foxp2 (p2/p2), myc-tagged Foxp1Δleu mutant and FLAG-tagged Foxp1 (p1Δleu/p1), myc-tagged Foxp2Δleu mutant and FLAG-tagged Foxp2 (p2Δleu/p2), myc-tagged Foxp4Δleu mutant and FLAG-tagged Foxp4 (p4Δleu/p4), myc-tagged Foxp4Δleu mutant and FLAG-tagged Foxp2 (p4Δleu/p2), myc-tagged Foxp4Δleu mutant and FLAG-tagged Foxp1 (p4Δleu/p1), myc-tagged Foxp2Δleu mutant and FLAG-tagged Foxp1 (p2Δleu/p1) expression plasmids. (D) Western blots of sample cell lysate showing expression of wild-type Foxp1 and Foxp1Δleu proteins. Lysates from pCMVTag3B-transfected (pCMVTag), myc-tagged Foxp1Δleu mutant plasmid-transfected (p1Δleu/myc), FLAG-tagged Foxp1Δleu mutant plasmid-transfected (p1Δleu/FLAG), and myc-tagged Foxp1Δleu mutant and FLAG-tagged Foxp1 plasmid-transfected (p1Δleu/p1) HEK-293 cells. The arrows in panels A and C indicate the 70- to 75-kDa bands of full-length Foxp1, Foxp2, and Foxp4 proteins. The antibodies used for each Western blot panel are indicated to the right in panels B and D.

motif eliminated DNA binding. The following two possibilities could explain these results: (i) the ΔE mutations change the conformation of the full-length Foxp1, Foxp2, and Foxp4 proteins such that they are no longer able to bind DNA, or (ii) dimerization is required for DNA binding. To distinguish between these two possibilities, a GST fusion protein encoding aa 457 to 714 of Foxp2 which lacks the leucine zipper motif was used in EMSA with the Fox DNA binding site from the mouse CC10 promoter. Of note, GST exists as a natural dimer in solution (18, 26, 28). The GST-Foxp2 fusion protein bound well to this oligonucleotide, even though it lacked the entire dimerization motif (Fig. 9C and D). However, upon cleavage of the GST moiety from the Foxp2 protein, DNA binding was lost. These data suggest that dimerization is required for DNA binding of Foxp1, Foxp2, and Foxp4 and that the natural dimerization of GST is able to rescue the DNA binding activity of a truncated Foxp2 protein that lacks the leucine zipper motif.

DISCUSSION

In our previous studies, we showed that Foxp1 and Foxp2 were transcriptional repressors expressed in lung, neural, and gut tissues (33). We have also reported that Foxp4 is expressed in an overlapping pattern with Foxp1 and Foxp2 in lung, gut, and neural tissues (21). In the present study, we have demon-

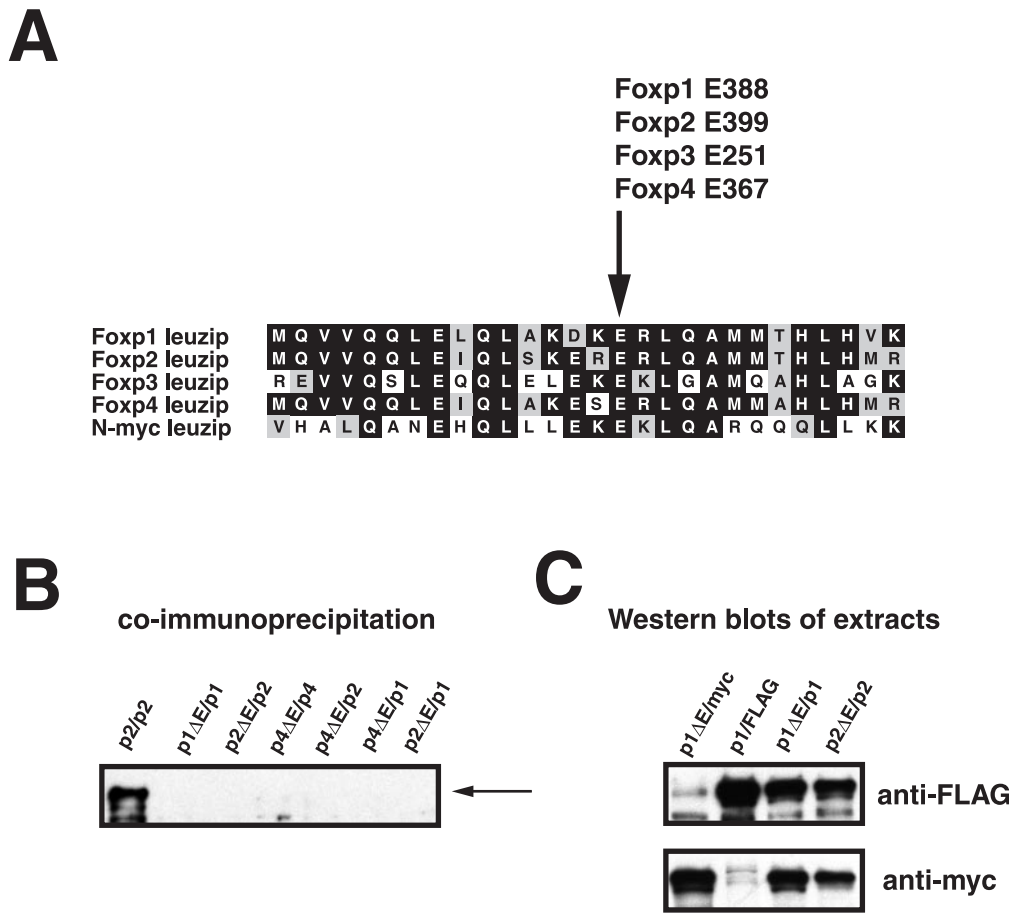

FIG. 7. Deletion of a single glutamic acid eliminates homo- and heterodimerization of Foxp1, Foxp2, and Foxp4. (A) Alignment of the leucine zipper (leuzip) motifs in Foxp1, Foxp2, Foxp3, Foxp4, and N-myc proteins, showing the conservation of the glutamic acid that is deleted in certain IPEX patients. Identical amino acids (white letters on black background) and similar amino acids (light gray shaded background) are indicated. (B) Coimmunoprecipitation assays using Foxp1, Foxp2, and Foxp4 ΔE mutants. Results are shown as described in the legend to Fig. 6, with the myc-tagged protein listed first and the FLAG- tagged protein listed second. The c-myc monoclonal antibody was used for immunoprecipitations, while the anti-FLAG monoclonal antibody was used on Western blots for immunodetection of coimmunoprecipitated proteins. Cells were transfected with myc-tagged Foxp2 and FLAG-tagged Foxp2 (p2/p2), myc-tagged Foxp1ΔE mutant and FLAG-tagged Foxp1 (p1ΔE/p1), myc-tagged Foxp2ΔE mutant and FLAG-tagged Foxp2 (p2ΔE/p2), myc-tagged Foxp4ΔE mutant and FLAG-tagged Foxp4 (p4ΔE/p4), myc-tagged Foxp4ΔE mutant and FLAG-tagged Foxp2 (p4ΔE/p2), myc-tagged Foxp4ΔE mutant and FLAG-tagged Foxp1 (p4ΔE/p1), myc-tagged Foxp2ΔE mutant and FLAG-tagged Foxp1 (p2ΔE/p1) plasmids. (C) Western blots of sample cell lysate showing expression of wild-type Foxp1, Foxp2, Foxp1ΔE, and Foxp2ΔE proteins. Lysates from myc-tagged Foxp1ΔE mutant plasmid-transfected (p1ΔE/myc), FLAG-tagged Foxp1 plasmid-transfected (p1/FLAG), myc-tagged Foxp1ΔE mutant and FLAG-tagged Foxp1 (p1Δleu/p1) plasmid-transfected, and myc-tagged Foxp2DE mutant and FLAG-tagged Foxp2 plasmid-transfected (p2ΔE/p2) HEK-293 cells. The arrow indicates the 70- to 75-kDa bands of full-length Foxp1, Foxp2, and Foxp4 proteins.

strated that Foxp1, Foxp2, and Foxp4 possess a complex molecular structure that regulates their ability to repress transcription and bind DNA by mediating homo- and heterodimerization and interactions with corepressor molecules, such as CtBP-1.

Characterization of the N-terminal region found to harbor the transcriptional repression activity of Foxp1, Foxp2, and Foxp4 revealed that there are at least two independent subdomains required for this activity. Subdomain 1 contains a leucine zipper motif that is conserved in all three proteins. Subdomain 2 contains a binding motif for the corepressor protein CtBP-1 and is found in Foxp1 and Foxp2 but not in Foxp4. CtBP-1 has been shown to bind to many different transcription factors and chromatin remodeling enzymes, including FOG-1/2, ZEB, and members of the class II histone deacetylases (12, 13, 15, 27, 32, 39). CtBP-1 has also been shown to homodimerize with itself, thus providing a bridge between DNA binding transcriptional regulators and other factors (35). Recently, mice harboring null alleles for CtBP-1 and the related molecule CtBP-2 have been reported (16). Interestingly, CtBP-1 null mice are viable, although smaller than their wild-type and heterozygous littermates. CtBP-2 null mice die at approximately embryonic day 10.5 and exhibit defects in both cardiovascular and neural development. Combinations of CtBP-1 and CtBP-2 null alleles produce dosage-dependent defects in a variety of tissues, including skeletal muscle, neural tube, heart, and vasculature. These data suggest that CtBP-1 and CtBP-2 have both distinctive and redundant functions in regulating gene transcription.

Deletion of the CtBP-1 site in subdomain 2 of Foxp2 re-

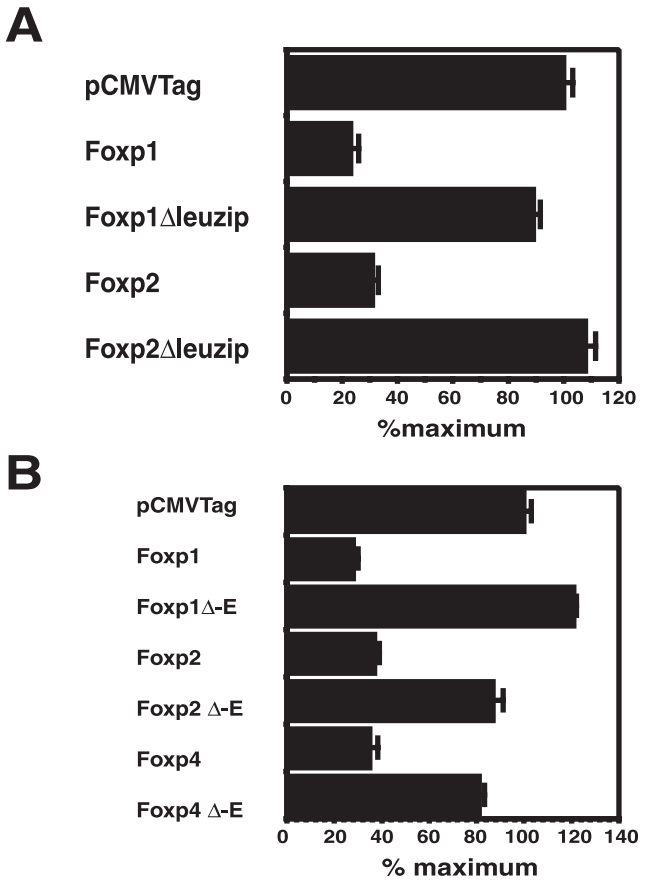

FIG. 8. Disruption of the leucine zipper motif in Foxp1, Foxp2, and Foxp4 results in loss of transcriptional repression. (A) H441 cells were transfected with the pCC10.luc reporter plasmid along with the pCMVTag3B control plasmid (pCMVTag), wild-type Fopx1 (Foxp1), Foxp1Δleu mutant (Foxp1Δleuzip), wild-type Foxp2 (Foxp2), or Foxp2Δleu mutant (Foxp2Δleuzip). Forty-eight hours after transfection, cells were harvested, and luciferase activity was measured and normalized to the activity obtained after transfection with the pCMVTag3B plasmid. (B) H441 cells were transfected with the pCC10.luc reporter along with the pCMVTag3B control plasmid (pCMVTag) and with a plasmid encoding wild-type Foxp1 (Foxp1), Foxp1ΔE mutant (Foxp1Δ-E), wild-type Foxp2 (Foxp2), Foxp2ΔE mutant (Foxp2Δ-E), wild-type Foxp4 (Foxp4), or Fopx4ΔE mutant (Foxp4Δ-E) protein. All assays included the pMSVβgal control plasmid, and differences in transfection efficiencies were corrected using a commercial β-galactosidase assay. Assays were performed in triplicate, and the data are presented as the maximum percentage of relative luciferase activity obtained upon cotransfection of the pCC10.luc reporter plasmid with the pCMVTag3B plasmid ± standard error of the mean.

sulted in the loss of transcriptional repression by this domain. However, mutation of this site in the full-length Foxp1 and Foxp2 proteins did not appreciably affect repression by the full-length proteins on the mouse CC10 promoter. This finding suggests that CtBP-1 binding is not essential for Foxp1 and Foxp2 transcriptional repression. Alternatively, subdomains 1 and 2 may be functionally redundant in their ability to repress transcription in these assays. Thus, the importance of repression mediated through the CtBP-1 binding motif may not be revealed in the presence of an intact subdomain 1. This is supported by our data demonstrating the importance of the CtBP-1 binding domain in the context of the subdomain 2 GAL4 fusion protein. The abilities of CtBP-1 to homodimerize and to recruit other transcriptional regulators may allow it to play an essential role in modulating Foxp1 and Foxp2 function in specific cell and developmental contexts not revealed in these studies.

Foxp1, Foxp2, Foxp3, and Foxp4 proteins have recently been implicated in several important human disorders and diseases, including a familial form of a rare speech disorder (Foxp2) and the immunological disease IPEX (Foxp3) (4, 7, 20). Foxp2 mutations in humans cause severe defects in the ability to articulate speech, part of which may be due to control of craniofacial muscles (20). Since Foxp2 is not expressed in skeletal muscle, these data suggest that Foxp2 may regulate specific neural circuits important for speech articulation or facial muscle control in humans. The observation that all three genes are expressed in distinct patterns during brain development also supports a role for this Fox subfamily in regulating neural development (21). Recent evidence has demonstrated that Foxp2 has gone through a very recent evolutionary change in humans (14, 41). Two amino acid changes have been identified in humans and chimpanzees, our most closely related primate cousin. Interestingly, these two changes lie outside the domains that we have identified in the present study, suggesting that these changes are not essential to either repression or dimerization by Foxp2. It will be important in the future to carefully characterize the importance of these amino acid changes and the effect they have on Foxp2 function.

Our data show that Foxp1, Foxp2, and Foxp4 are the first Fox proteins identified that regulate DNA binding and transcription through dimerization, which is mediated by a conserved leucine zipper motif found in each protein. Surprisingly, a single amino acid deletion in this region, which is identical to a Foxp3 mutation found in patients with IPEX (9), eliminates the abilities of Foxp1, Foxp2, and Foxp4 to dimerize, bind to DNA, and repress transcription. Intriguingly, the addition of a GST moiety to the forkhead DNA binding domain of Foxp2 rescues its ability to bind to DNA. Since GST is an obligate dimer in solution, this further supports evidence showing that dimerization is required for Foxp1, Foxp2, and Foxp4 DNA binding (18, 26, 28). Other Fox proteins have been shown to bind to DNA as obligate monomers, and the winged-helix DNA binding domain found in all Fox proteins has been shown to exist as a monomer in solution (10). Although the winged-helix domain of Foxp1, Foxp2, and Foxp4 is somewhat different from that of other Fox proteins, it still contains all of the conserved amino acid residues required for DNA binding, and mutations in these conserved residues disrupt DNA binding. In addition, Foxp1, Foxp2, and Foxp4 are capable of binding to the consensus Fox DNA binding site found in the mouse CC10 promoter, and DNA binding site selection using Foxp2 resulted in the isolation of consensus Fox protein DNA binding sites (data not shown). However, the differences between the winged-helix domains of Foxp1, Foxp2, and Foxp4 and other Fox proteins may impart unique characteristics, including the requirement of dimerization for DNA binding. This dimerization may allow for an extra level of posttranslational regulation and DNA site recognition by Foxp1, Foxp2, and Foxp4 where specific combinations of homo- and heterodimers control transcription of downstream target genes.

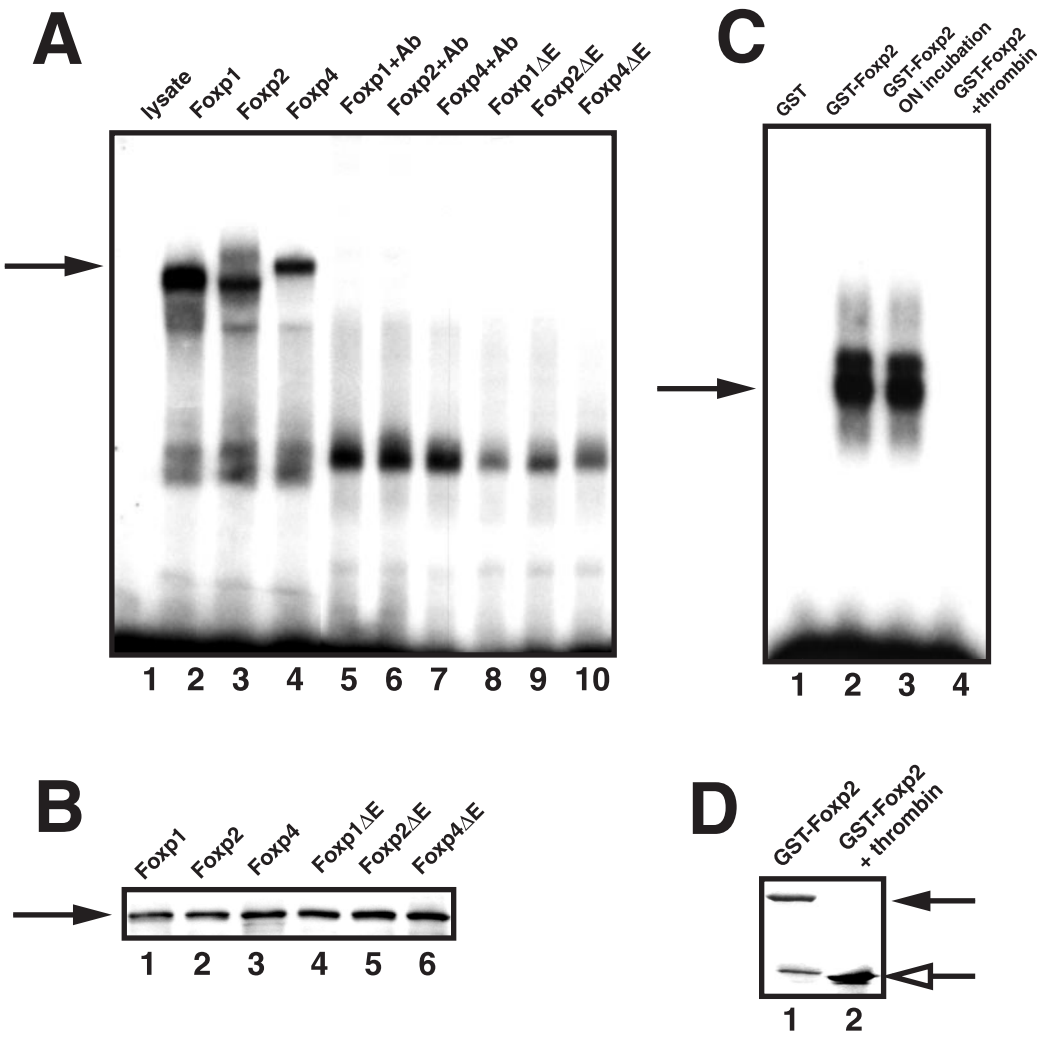

FIG. 9. Dimerization is required for Foxp1, Foxp2, and Foxp4 proteins to bind DNA. The Fox DNA binding consensus site in the mouse CC10 promoter was used to perform EMSA on in vitro-translated Foxp1, Foxp2, and Foxp4 or GST-Foxp2 fusion proteins. (A) EMSA on unprogrammed cell lysate (lane 1) and cells treated with wild-type Foxp1, Foxp2, and Foxp4 alone (lanes 2 to 4) and with specific polyclonal antibodies (Ab) to each protein (lanes 5 to 7), and with mutant Foxp1, Foxp2, and Foxp4 ΔE proteins (lanes 8 to 10). Wild-type Foxp1, Foxp2, and Foxp4 DNA binding was eliminated with specific polyclonal antibodies to each protein (lanes 5 to 7). (B) Western blot of in vitro-translated wild-type Foxp1, Foxp2, and Foxp4 and Foxp1, Foxp2, and Foxp4 ΔE mutations. The same amount of in vitro-translated protein used in the EMSA (5 μl) was resolved on a SDS-polyacrylamide gel, blotted, and probed with the anti-myc monoclonal antibody. The position of the Foxp1, Foxp2, and Foxp4 proteins is indicated by the arrow. (C) EMSA using the mouse CC10 Fox oligonucleotide and GST protein (lane 1), untreated GST-Foxp2 protein (lane 2), GST-Foxp2 protein incubated overnight (ON) without thrombin (lane 3), and GST-Foxp2 protein incubated overnight with thrombin (lane 4). The shifted band is indicated by the arrow. (D) SDS-polyacrylamide gel showing the integrity of GST-Foxp2 (lane 1) and cleaved GST-Foxp2 (lane 2) proteins. The black arrow indicates the full-length GST-Foxp2 fusion protein, and the white arrow indicates the cleaved GST and Foxp2 portions of the fusion protein, which comigrate closely on the gel.

Transcriptional repression through the leucine zipper motif in the GAL4 heterologous DNA binding system also requires the integrity of the leucine zipper motif. One possible explanation for this is that the dimerized leucine zipper motif may produce a certain conformational interface that recruits other corepressor molecules. There are several precedents for this, including interactions between SMRT/N-CoR and the transcriptional repressor ETO and the interactions between KRAB domain-containing zinc finger proteins and the KAP-1 corepressor (25, 40). In both of these cases, oligomerization is required for interaction of the corepressor proteins, possibly by providing a unique protein interaction interface that does not exist in the monomeric proteins. In this context, it will be important in the future to ascertain the protein structure of this dimerization domain and determine both the similarities and differences from other leucine zipper motifs.

The region of Foxp1, Foxp2, and Foxp4 containing the dimerization motif also contains several other motifs involved in protein-protein interactions. Our data show that the conserved zinc finger is not required for transcriptional repression. Indeed, when fused to the GAL4 DNA binding domain, the zinc finger motif of Foxp2 actually activates transcription more than threefold (data not shown). Thus, it is likely that the entire N-terminal region of Foxp1, Foxp2, and Foxp4 acts as a

complex docking site for multiple transcriptional cofactors, potentially both corepressors and coactivators, allowing Foxp1, Foxp2, and Foxp4 to differentially regulate tissue-specific gene transcription through common DNA binding sequences.

The ability of Foxp1, Foxp2, and Foxp4 proteins to heterodimerize could add great flexibility to how these factors can regulate gene transcription. Results from several labs, including our own, have shown that Foxp1, Foxp2, and Foxp4 are coexpressed in several tissues, including the lung and brain, suggesting possible redundancy in regulating certain target genes (3, 21, 33). The precise combination of homodimers and heterodimers may provide an elegant means by which Foxp1, Foxp2, and Foxp4 can regulate gene transcription during development. Identification of transcriptional targets of each of these factors should be helpful in determining how the dimerization abilities of Foxp1, Foxp2, and Foxp4 affect gene expression.

Although other transcriptional regulators, such as members of the basic helix-loop-helix and bZIP families, regulate gene transcription through dimerization, to our knowledge, Foxp1, Foxp2, and Foxp4 are the first Fox proteins that have been shown to require dimerization for activity. In addition, Foxp1, Foxp2, and Foxp4 are the first Fox proteins that have been shown to bind to the corepressor CtBP-1, and this interaction illustrates the complex nature with which these factors regulate gene transcription. Future studies characterizing additional cofactors, especially proteins which interact uniquely with the dimerization domain, should provide critical insights into the complex mechanisms underlying how Foxp1, Foxp2, and Foxp4 regulate gene transcription.

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