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Analysis of FOXP3 Reveals Multiple Domains Required for Its Function as a Transcriptional Repressor¹

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Foxp3 has been shown to be both necessary and sufficient for the development and function of naturally arising CD4⁺CD25⁺ regulatory T cells in mice. Mutation of *Foxp3* in *Scurfy* mice and *FOXP3* in humans with IPEX results in fatal, early onset autoimmune disease and demonstrates the critical role of FOXP3 in maintaining immune homeostasis. The FOXP3 protein encodes several functional domains, including a C2H2 zinc finger, a leucine zipper, and a winged-helix/forkhead (FKH) domain. We have shown previously that FOXP3 functions as a transcriptional repressor and inhibits activation-induced IL-2 gene transcription. To characterize the role of each predicted functional domain on the in vivo activity of FOXP3, we have evaluated the location of point mutations identified in a large cohort of patients with the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) and found them to cluster primarily within the FKH domain and the leucine zipper, but also present within the poorly defined N-terminal portion of the protein. The molecular functions of each of the IPEX-targeted domains were investigated. We show that FOXP3 is constitutively localized to the nucleus and this localization requires sequences at both the amino and C-terminal ends of its FKH domain. Moreover, FOXP3 was found to homodimerize through its leucine zipper. We also identify a novel functional domain within the N-terminal half of FOXP3, which is required for FOXP3-mediated repression of transcription from both a constitutively active and a NF-AT-inducible promoter. Furthermore, we demonstrate that IPEX mutations in these domains correlate with deficiencies in FOXP3 repressor function, corroborating their in vivo relevance. *The Journal of Immunology*, 2006, 177: 3133–3142.

The forkhead (FKH)⁵ box protein FOXP3 is a member of the P subfamily of Fox transcription factors, which as a group are characterized by the presence of a highly conserved winged-helix/FKH DNA binding domain (DBD). Studies in model organisms such as *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Mus musculus* have demonstrated a pivotal role for Fox family transcription factors in embryonic patterning, development, and metabolism (1). Recent elucidation of roles for this family of transcriptional regulators in thymic development (*Foxn1*), lineage commitment (*Foxp3*), and function of lymphocytes (*Foxj1*, *Foxo3*, and *Foxd1*) has firmly established a role for

this transcription factor family in the development and maintenance of normal immune responses (2, 3).

Foxp3 was identified as the gene mutated in the *Scurfy* mouse, a spontaneously arising strain in which affected males are characterized by wasting, exfoliative dermatitis, lymphadenopathy, hepatosplenomegaly, multiorgan lymphocytic infiltrates, and the presence of autoantibodies (4, 5). *FOXP3* was subsequently found to be the gene mutated in the human immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), which is phenotypically similar to *Scurfy* with affected males displaying systemic autoimmune disease affecting bowel, skin, endocrine organs, and blood (6–9). In both *Scurfy* and IPEX, the defects in *Foxp3* are fatal, leading to death by 3 wk and 1–2 years, respectively. Recent data suggest that the mechanism by which defects in *Foxp3* lead to overwhelming autoimmunity is through a lack of naturally arising regulatory T cells (T_{regs}) (10–14).

Murine studies have been particularly revealing with respect to the importance of Foxp3 for the regulation of autoreactive T cells in the periphery. However, relatively little is known about the molecular mechanism by which FOXP3 functions, particularly in humans. We have demonstrated previously that FOXP3 acts as a transcriptional repressor when expressed in either nonlymphoid cells or T cell lines, where it inhibits activation-induced cytokine expression (15). The *Foxp3* mutation present in *Scurfy* mice results in the loss of the C-terminal FKH domain, suggesting the importance of this domain for Foxp3 function (5). Structurally, FOXP3 shares similar protein architecture with other members of the Foxp subfamily with a C2H2 zinc finger, a leucine zipper, and a FKH domain. In Foxp1, Foxp2, and Foxp4, these domains are centrally located, whereas in FOXP3, the zinc finger and leucine zipper domains are centrally located, but the FKH domain is at the C terminus. In addition, FOXP3 contains a functionally undefined N terminus that is rich in proline. The role of these other predicted

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⁵ Abbreviations used in this paper: FKH, forkhead; CtBP1, C-terminal binding protein 1; DBD, DNA binding domain; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome; NLS, nuclear localization signal; T_{reg}, regulatory T cell; wt, wild type.

functional domains in the repressor activity of FOXP3 is yet unknown.

In an effort to define the domains of human FOXP3 that are important for its function in vivo, we have sequenced the *FOXP3* gene in a large cohort of patients with the phenotypic features of IPEX syndrome. Among the cohorts reported by our group and others, mutations affecting a single amino acid were present in 22 patients (8, 16–18) (T. Torgerson and H. Ochs, manuscript in preparation). Interestingly, the identified mutations are predominantly located in three regions of the FOXP3 protein: the FKH DBD, the leucine zipper, and an area of the N-terminal region with unknown function (Fig. 1). We therefore set out to characterize the functions of the IPEX-targeted domains of FOXP3 and then validate our findings by correlating IPEX-targeted mutations with deficiencies in FOXP3 function. Using this approach, we have identified the sequences of FOXP3 required for nuclear import and have determined that FOXP3 can homodimerize, a process dependent on the presence of a functional leucine zipper. Furthermore, we have identified a N-terminal domain of FOXP3 that is both necessary and sufficient for the repression of a constitutively active luciferase reporter. A slightly larger N-terminal region of FOXP3 is also sufficient to repress transcription from a NF-AT-inducible luciferase reporter. The identification of missense mutations in the FKH domain, leucine zipper, and a N-terminal region of FOXP3 in patients with IPEX syndrome provides strong supportive evidence for the relevance of our findings in vivo.

Materials and Methods

Constructs

Full-length FOXP3 expression plasmids have been described previously (13).

GFP and V5-tagged FOXP3 constructs. Fragments of the *FOXP3* cDNA were amplified by PCR using *FOXP3*-specific primers that also encoded recombination sites for use with Gateway vectors (Invitrogen Life Technologies). Amplified fragments were recombined into pDONR201 using BP clonase (Invitrogen Life Technologies) to create entry clones. All clones were verified by sequencing and then recombined into either pcDNA3.1/nV5DEST (V5 tagged) or pcDNA-DEST53 (GFP tagged) using LR clonase (Invitrogen Life Technologies). IPEX point mutants were generated in the full-length, wild-type (wt) *FOXP3* cDNA entry clone using the Quickchange site-directed mutagenesis system (Stratagene) with paired sense and antisense primers encoding the desired mutation. All mutations were confirmed by sequencing.

Gal4 DBD fusion constructs. Fragments of the *FOXP3* cDNA were generated by PCR using specific primers that introduced *EcoRI* sites at each end. The resulting PCR products were ligated into the pGEM-T cloning vector (Promega), verified by sequencing, and subsequently digested with *EcoRI* and ligated in frame into the Gal4 DBD expression vector (19) (provided by E. Morrisey, University of Pennsylvania, Philadelphia, PA).

Untagged FOXP3 constructs. The wt or mutant *FOXP3* cDNAs were amplified by PCR with specific primers that introduced *EcoRI* sites at each end. These were subsequently digested with *EcoRI* and ligated into the internal ribosome entry site 2-enhanced GFP expression vector (Clontech).

Luciferase reporter vectors. The SV40 Gal4 luciferase reporter contains five copies of the yeast Gal4 DBD-binding sequence upstream of the SV40 promoter and has been described previously (20). The Gal4/NF-AT luciferase reporter was generated by PCR amplification of five copies of the Gal4 DBD-binding sequence using specific primers that introduce *HindIII* sites at each end. The product was ligated upstream of three copies of the NF-AT-binding sequence in the NF-AT luciferase reporter described previously (15). The IL-2 reporter construct containing 715 bp of the human IL-2 promoter driving firefly luciferase was obtained from Panomics. The 3× NF-AT and 3× STAT1 firefly luciferase reporters were obtained from Panomics. The control pRL-TK vector containing *Renilla* luciferase under the control of the constitutively active thymidine kinase promoter was obtained from Promega.

Cell lines

HEK 293 T cells were maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% FBS, 1% penicillin, and 1% streptomycin. The T lymphoma cell line EL4 was maintained in RPMI 1640 (Invitrogen

Life Technologies) supplemented with 10% FBS, 1% penicillin, and 1% streptomycin.

A HEK 293 cell line that inducibly expresses V5-tagged wt FOXP3 in response to tetracycline (293/Tet/wtFP3) was generated by cotransfection of the pcDNA5/FRT/TO vector (Invitrogen Life Technologies) bearing a cDNA encoding N-terminal V5 epitope-tagged wt FOXP3 (aa 2–431) and the pOG44 plasmid (Invitrogen Life Technologies) encoding Flp recombinase into the Flp-In T-Rex 293 cell line (Invitrogen Life Technologies). Forty-eight hours after transfection, cells were selected and maintained in blasticidin (15 μg/ml) and hygromycin (100 μg/ml). Inducible expression of FOXP3 in the cell line was confirmed by both quantitative real-time PCR and Western blot.

Transfections and reporter gene assays

Expression constructs encoding wt, truncated, or mutated FOXP3 were either transfected alone or cotransfected with the GAL4 luciferase reporter and the β-galactosidase reporter into HEK 293 T cells. A total of 200 ng of each plasmid (or as otherwise indicated) was mixed with Fugene6 transfection reagent (Roche) in DMEM supplemented with 1% penicillin and 1% streptomycin, allowed to incubate for 15 min, then added to subconfluent cells grown in 24-well plates. Twenty-four hours posttransfection, cells were washed using 1× PBS, and lysates were prepared in 1× reporter lysis buffer, according to the manufacturer's specifications (Promega). Each transfection was performed in triplicate. Cell lysates were assayed in duplicate for luciferase activity using the Luciferase Assay System (Promega) and an EG & G Berthold Lumat 9507 luminometer (PerkinElmer Life Sciences), according to the manufacturers' specifications. The β-galactosidase activity in each lysate was determined using the β-galactosidase enzyme assay system (Promega) and used as a control for transfection efficiency to normalize the measured luciferase activity. Expression of each FOXP3-Gal4 DBD fusion protein was confirmed by Western blotting using an anti-Gal4 DBD rabbit polyclonal Ab (Sigma-Aldrich) or an anti-Gal4 DBD mouse mAb sc510 (Santa Cruz Biotechnology).

Transfections in EL4 cells were done by electroporation in a Bio-Rad Gene-Pulser (Bio-Rad). Each FOXP3 expression construct was cotransfected with the GAL4 luciferase reporter and the β-galactosidase reporter. A total of 1×10^7 cells was mixed with 30 μg (10 μg/construct) of plasmid DNA in RPMI 1640 supplemented with 1% penicillin and 1% streptomycin and electroporated at 250 V in 4-mm cuvettes. Cells were cultured in complete RPMI 1640 supplemented with 10% FBS, 1% penicillin, and 1% streptomycin for 16–20 h, then 1×10^6 viable cells were plated into 24-well plates and stimulated for 6 h with PMA (25 ng/ml) and ionomycin (1.5 μM) or vehicle control (DMSO). After stimulation, cells were washed in 1× PBS, lysates were prepared, and luciferase activity was measured, as described above. Differences in luciferase values were tested for statistical significance using an unpaired Student's *t* test.

Transfections of Jurkat T cells were performed using Superfect (Qiagen). Unless otherwise indicated, 2.5×10^6 cells were transfected with 2 μg of the indicated firefly luciferase reporter vector (IL-2, 3× NF-AT, or 3× STAT1), 100 ng of the pRL-TK *Renilla* luciferase control vector, and 1 μg of the indicated FOXP3 expression vector or empty control vector. Eighteen hours after transfection, cells were divided, and half were stimulated with PMA (5 nM) and ionomycin (2 μM), while the other half were treated with vehicle (DMSO) control for 16 h. Luciferase assays were run using the Dual Luciferase Assay kit (Promega), per the manufacturer recommendations. Luminescence was read in white 96-well plates using a Victor3 multilabel plate reader (PerkinElmer) with autoinjectors.

Immunoprecipitations

The 293/Tet/wtFP3 cell line (see above) was transfected with constructs encoding a panel of domain-deleted or point-mutated FOXP3 proteins tagged at the N terminus with GFP. Transfections were performed, as indicated. Six hours after transfection, cells were treated with tetracycline (1 μg/ml) to induce the V5-tagged wt FOXP3. Twenty-four hours after transfection, whole cell extracts were prepared in radioimmunoprecipitation assay buffer containing 1% mammalian cell protease inhibitor mixture (Sigma-Aldrich). Extracts were cleared by centrifugation at high speed for 10 min at 4°C. Supernatants were incubated with 1 μg of rabbit polyclonal anti-GFP Ab (Molecular Probes) per sample for 1 h at 4°C, and then immunoprecipitates were recovered on 25 μl of protein G-Sepharose (Pierce). Beads were washed five times with cold radioimmunoprecipitation assay buffer, boiled in SDS-PAGE sample buffer, and run on an 8% polyacrylamide gel. Western blots were performed and probed for the presence of the coimmunoprecipitated V5-tagged FOXP3 protein using anti-V5 mAb conjugated to HRP (Invitrogen Life Technologies) at a dilution of 1/5000. As a control, whole cell extract was probed by Western blot for the presence of the induced V5-FOXP3 protein (using anti-V5 mAb) or the various

GFP-FOXP3 fusion proteins using the anti-GFP Ab (as above) at a dilution of 1/1000.

Results

FOXP3 mutations identified in patients with IPEX syndrome suggest functionally important domains of the protein

The locations of the IPEX-associated point mutations in FOXP3 were plotted adjacent to the locations of the predicted functional domains of FOXP3 (Fig. 1) (8, 16–18) (T. Torgerson and H. Ochs, manuscript in preparation). As shown, mutations are predominantly located in and around the FKH DBD, in the leucine zipper, and in the proline-rich N-terminal half of the protein. The prevalence of mutations within these regions highlights the functional importance of these domains *in vivo*. Interestingly, no mutations have been identified in the zinc finger domain, suggesting that it may not play a vital role in FOXP3 function.

Sequences at both the N- and C-terminal portion of the FKH domain contribute to FOXP3 nuclear import

FOXP3 has been shown previously to constitutively localize to the nucleus both in cells transiently transfected with FOXP3-encoding plasmids (15) and in the T cells of mice bearing GFP-tagged Foxp3 expressed from the endogenous Foxp3 promoter (13). To date, there is no evidence that this process is regulated as it is for other transcription factors such as NF- κ B or NF-AT (21).

To identify the specific sequences required for targeting of FOXP3 to the nucleus and to determine whether any of a panel of mutations identified in IPEX patients affects nuclear import, we generated N-terminal GFP-FOXP3 fusion proteins with a series of overlapping deletion mutants and point mutations within the FOXP3 sequence. The constructs were transfected into HEK 293 cells and evaluated by fluorescence microscopy for the subcellular localization of the GFP-FOXP3 fusion proteins. A C-terminal fragment of FOXP3 containing the entire FKH DBD with short flanking sequences at each end was found to be both necessary and sufficient for import of FOXP3 to the nucleus (Fig. 2). Mutation of two basic amino acids to acidic amino acids (K415/416E) within a putative nuclear localization signal (NLS) (₄₁₄RKKR₄₁₇) near the C-terminal end of the FKH domain abrogates nuclear import of FOXP3 (Fig. 2). Interestingly, no mutations affecting this NLS have been identified in patients with IPEX. Screening of a panel of point mutations identified in patients with IPEX identified only one that affected the constitutive nuclear import of FOXP3 (R347H), and this was only partial with the majority of FOXP3 being localized to the nucleus, but a portion remaining in the cytoplasm (Fig. 2 and data not shown). The identification of regions at both ends of the FKH domain that are involved in nuclear targeting of FOXP3 is consistent with the pattern observed in another FKH family member, hepatocyte NF-3 β (22).

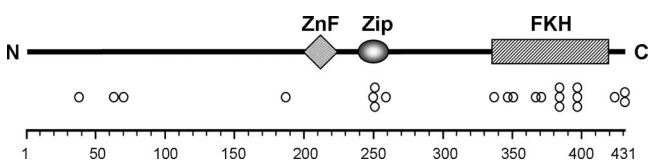


FIGURE 1. FOXP3 mutations identified in IPEX patients are located in and around predicted structural domains. Locations of identified point mutations in FOXP3 were plotted adjacent to a linear representation of the FOXP3 protein, showing the location and sizes of the predicted structural domains. Numbers along the *bottom* of the graph indicate amino acid number (1–431). Each circle represents a mutation identified in a single kindred. ZnF, zinc finger; Zip, leucine zipper; FKH, FKH DBD.

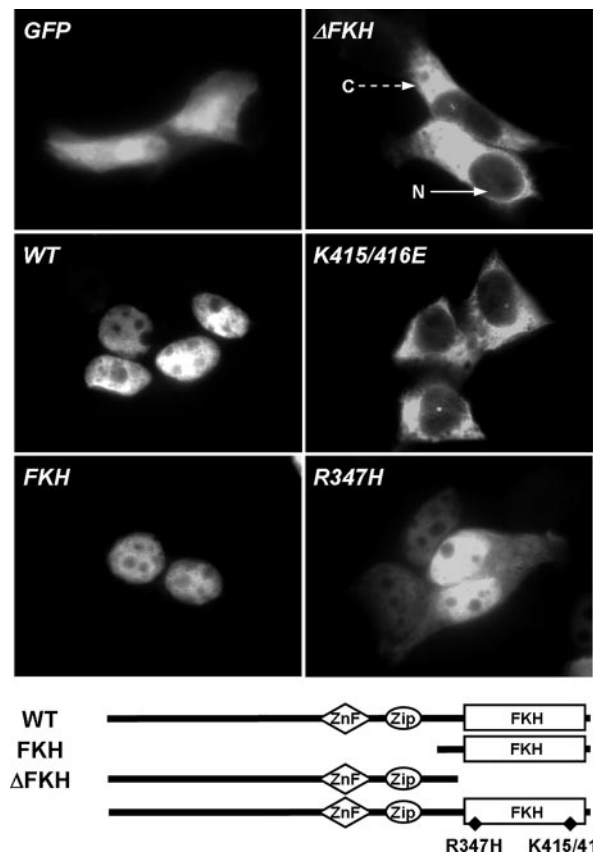


FIGURE 2. Sequences required for nuclear import of FOXP3. Constructs encoding N-terminal GFP-FOXP3 fusion proteins as indicated were transiently transfected into HEK 293 cells and evaluated by standard fluorescence microscopy for the ability to translocate to the nucleus. GFP by itself is below the size-exclusion limit of the nuclear pore complex, and therefore is distributed throughout the cell. Solid arrow denotes the nucleus (N = nucleus), while the dashed arrow denotes the cytoplasm (C = cytoplasm).

FOXP3 homodimerizes through the leucine zipper domain

Most transcriptional regulators bind to DNA coordinately either in homomultimeric complexes or in concert with other factors. Consistent with this, Foxp subfamily members Foxp1, Foxp2, and Foxp4 have been shown to interact with one another either as homo- or heterodimers (20, 23). In addition, Foxp1 interacts with Foxp3 in *in vitro* assays, although the physiologic significance of this interaction *in vivo* is unknown (23). FOXP3 has significant sequence similarity to Foxp1, Foxp2, and Foxp4 within the FKH DBD domain and within the predicted C2H2 zinc finger and leucine zipper domains, which may both be important for protein-protein interaction. Outside of these regions, its sequence is quite divergent from the other FoxP subfamily members.

To determine whether FOXP3 is also capable of homodimerization, a coimmunoprecipitation approach using two differentially tagged FOXP3 proteins was used. A HEK 293 cell line (293/Tet/wtFP3) was generated that expresses N-terminal V5 epitope-tagged wt FOXP3 in response to tetracycline. This cell line was transfected with vectors encoding wt or mutant FOXP3 proteins tagged at the N terminus with GFP, resulting in the two differentially tagged FOXP3 proteins being expressed simultaneously. FOXP3 protein complexes were immunoprecipitated using an Ab to GFP and probed by Western blot for association with the V5-tagged wt protein. In this system, expression of the full-length

GFP-FOXP3 (GFP-wt) leads to coprecipitation of V5-FOXP3, indicating that FOXP3 is capable of homodimerization (Fig. 3). Expression of GFP-fusion proteins with truncated FOXP3 isoforms demonstrated that homodimerization was lost only with the FKH isoform (GFP-FKH), which lacks the zinc finger and leucine zipper domains (Fig. 3). Similarly, the isoform containing only the zinc finger and leucine zipper (GFP-ZnF/Zip) is capable of mediating homodimerization, demonstrating that this region is both necessary and sufficient for this activity (Fig. 3).

To evaluate whether the zinc finger, leucine zipper, or both are required for homodimerization, we used two point mutations, one that destroys the structure of the zinc finger (GFP-C204S) and a second, identified in four families with IPEX syndrome, that affects the function of the leucine zipper (GFP- Δ E251). Mutation of the homologous E in the *c-myc* leucine zipper abrogates its ability to mediate dimerization (24). In the case of FOXP3, only the mutation affecting the leucine zipper domain (GFP- Δ E251) resulted in loss of homodimerization (Fig. 3), indicating that the leucine zipper is both necessary and sufficient for this activity.

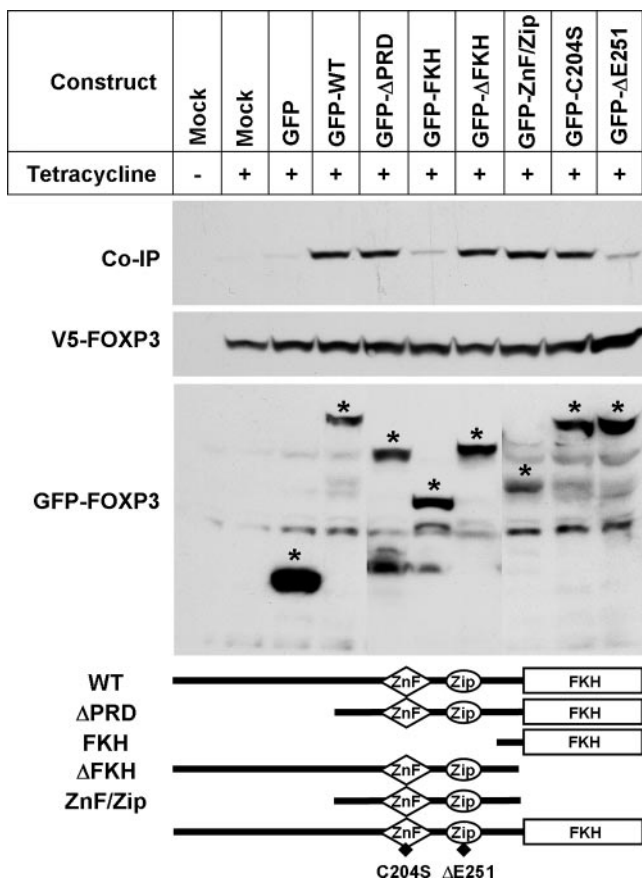


FIGURE 3. FOXP3 homodimerizes through the leucine zipper domain. The 293/Tet/wtFP3 cells were transfected with GFP-FOXP3 fusion constructs bearing various fragments or mutations of FOXP3 as shown (see *Materials and Methods*). Six hours posttransfection, V5-FOXP3 expression was induced. Twenty-four hours posttransfection, cells were lysed and complexes were immunoprecipitated with rabbit anti-GFP polyclonal Ab. Immunoprecipitates were washed, boiled in SDS sample buffer, and separated by SDS-PAGE. Western blots were probed for the presence of the wt V5-tagged FOXP3 (*top panel*). The tetracycline-inducible expression of V5-tagged wt FOXP3 was confirmed in whole cell lysates using anti-V5 Ab before immunoprecipitation (*middle panel*). Expression of the various GFP-FOXP3 fusion proteins was confirmed by Western blot in whole cell extracts before immunoprecipitation using rabbit polyclonal anti-GFP Ab (*bottom panel*).

The N-terminal region of FOXP3 is critical for transcriptional repression

The Foxp subfamily members Foxp1, Foxp2, and Foxp4 have transcriptional repressor function that is dependent on regions outside of the FKH DBD. Foxp1 and Foxp2 associate with the transcriptional corepressor C-terminal binding protein 1 (CtBP1), via a PXDLS-like consensus sequence located between the leucine zipper and FKH domains. Foxp4 lacks this consensus-binding sequence and consequently does not bind CtBP1 (20). A second domain located outside of the glutamine-rich region of the N-terminal half of Foxp1, Foxp2, and Foxp4 is also capable of mediating gene repression. Although FOXP3 bears marked sequence similarity to Foxp1, Foxp2, and Foxp4 within the conserved zinc finger, leucine zipper, and FKH domains, it has no significant similarity to either the PXDLS-like motif or the glutamine-rich region. To better define the regions of FOXP3 outside the FKH domain that are required to repress gene transcription from the constitutively active SV40 promoter, fragments of FOXP3 were fused to the Gal4 DBD (aa sequence 1–147 of Gal4). The Gal4 DBD sequence contains three domains and performs three separate functions; it contains a dimerization domain, a NLS, and a DNA binding domain (DBD) (25). These mutants were evaluated in HEK 293 T cells for their effect on the Gal4 luciferase reporter plasmid bearing Gal4 DNA binding sites adjacent to the constitutively active SV40 promoter (see *Materials and Methods* for details). Expression of wt FOXP3 fused to the Gal4 DBD (wt) results in ~90% reduction in transcriptional activity compared with expression of the Gal4 DBD by itself (Fig. 4A). Expression of a FOXP3 mutant lacking the FKH domain (Δ FKH) results in a level of transcriptional repression comparable with wt FOXP3 (Fig. 4A). This indicates that the Gal4 DBD is able to functionally mediate nuclear import, dimerization, and DNA binding in the absence of the FKH domain in this experimental system, allowing us to investigate the repressor function of other domains of the FOXP3 protein.

Expression of a Gal4-FOXP3 fusion protein containing the first 198 aa of FOXP3 (1–198) resulted in transcriptional repression equivalent to Δ FKH (Fig. 4A). In contrast, expression of a Gal4-FOXP3 fusion protein bearing the zinc finger and leucine zipper of FOXP3 (ZnF/Zip) shows no repressive effect on transcription. The inability of the ZnF/Zip domain to inhibit transcription was not due to the lack of protein expression, because its expression was confirmed by Western blot analysis in transfected HEK 293 T cells (data not shown). These results demonstrate that sequences in the N-terminal portion of FOXP3 are both necessary and sufficient to repress transcription.

To further define the repressor domain identified in the first 198 residues of FOXP3, overlapping fragments of this region were generated and used in the same assay. Constructs encoding aa 1–132 and 67–198 resulted in ~70 and ~60% inhibition of transcription, respectively (Fig. 4B). The decreased repressor function observed with each of these constructs suggests one of two possibilities: 1) that each fragment contains a repressor domain and that their effect is additive, or 2) that the region of overlap between the two constructs (aa 67–132) contains the repressor domain, and that some flanking sequence is required for full repressor function. To address these possibilities, constructs encoding each half of the N terminus (1–105 and 106–198) as well as the region of overlap (67–132) were generated. Both the N-terminal 1–105 and the overlap 67–132 constructs exhibited similar repressor activity (~50%), whereas the 106–198 construct was unable to exert any repressive effect (Fig. 4B). To more fully map the functional repressor domain, an additional construct was prepared encoding aa 67–105 of

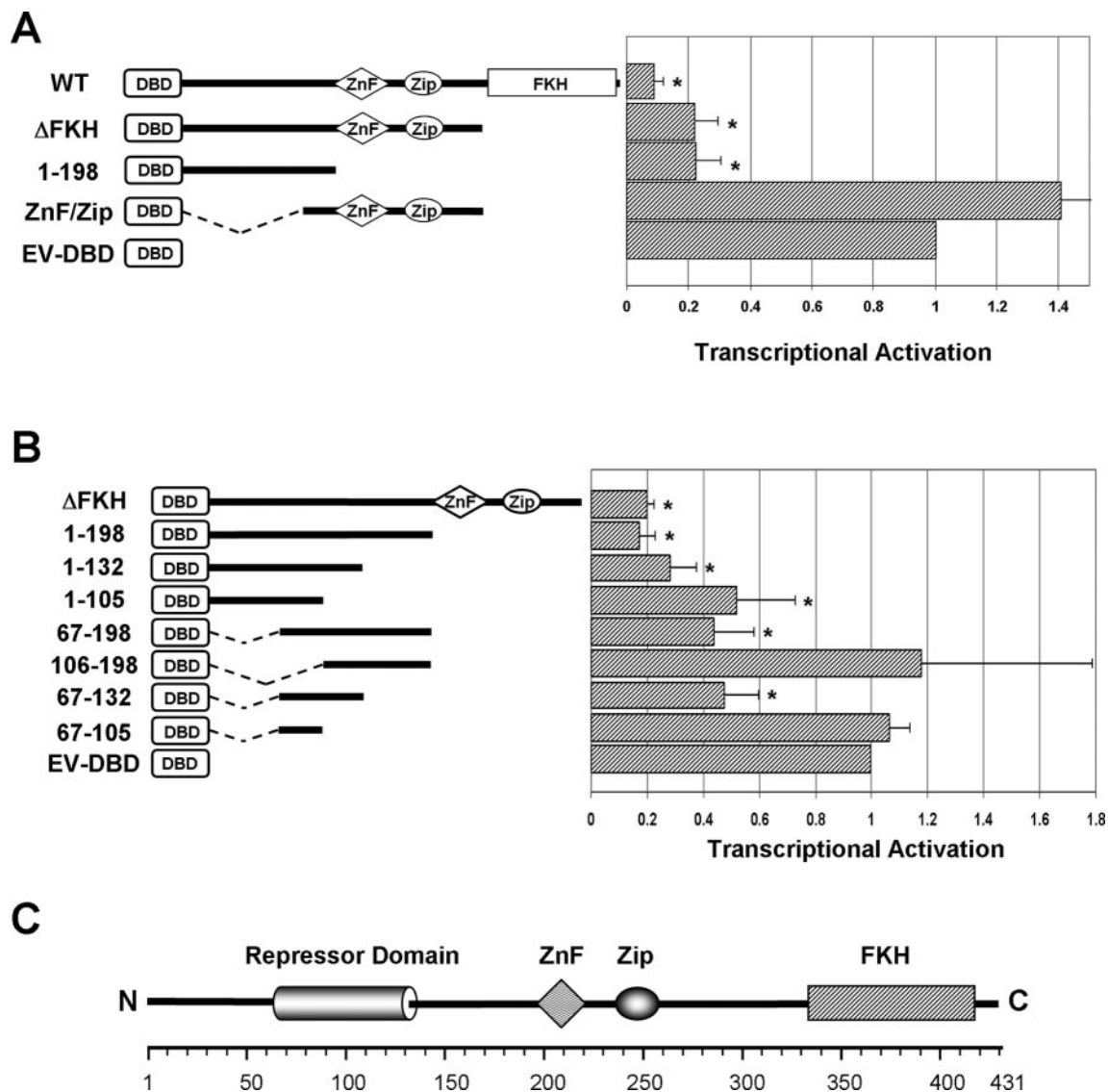


FIGURE 4. A N-terminal domain of FOXP3 is necessary and sufficient to repress transcription from a constitutively active promoter. *A* and *B*, Expression constructs encoding full-length or truncation mutants of FOXP3 fused to the DBD of GAL4 as shown were cotransfected into HEK 293 cells with the 5× GAL4 luciferase and β-galactosidase reporters. Luciferase assays were performed on lysates prepared 24 h posttransfection. Luciferase activity was normalized by β-galactosidase activity. The data are an average of three experiments, and the error bars represent the SD from the mean. *, Indicates reduction in activity relative to empty vector is statistically significant ($p < 0.05$). *C*, Linear representation of the FOXP3 protein indicating the position and size of the identified N-terminal repressor domain.

the FOXP3 protein. This fusion protein exhibited no repressor activity (Fig. 4*B*). The inability of the 67–105 and 106–198 Gal4-FOXP3 fusion proteins to inhibit transcription was not due to the lack of protein expression, because expression levels were similar to those of functional Gal4-FOXP3 fusion proteins (data not shown).

To demonstrate that differences in repression mediated by the different Gal4-FOXP3 fusion proteins were due to differences in the functionality of the encoded domain(s), and that the amount of transcriptional repression has not reached a plateau at the dose used in Fig. 4, decreasing amounts of Gal4-FOXP3 cDNA were transfected and the effects on transcription were observed. Decreasing the amount of cDNA resulted in a graded increase in transcription (data not shown). The 67–132 construct repressed transcription ~50% of the extent repressed by 1–198. This trend was observed at several different doses of cDNA. Altogether, these

experiments define the minimal repressor domain to be within aa 67–132 of the N-terminal portion of FOXP3 (Fig. 4*C*).

Domains of FOXP3 involved in inhibition of NF-AT-mediated transcriptional activation

The ectopic expression of FOXP3 in primary T cells and T cell lines has been shown to suppress NF-AT-mediated gene transcription and consequently to inhibit activation-induced cytokine expression, particularly of IL-2 (15, 26). The mechanism by which this occurs is not known. Because loosely conserved FKH DNA binding sites have been identified within, and adjacent to NF-AT binding sites in the promoters of cytokine genes, one hypothesis is that FOXP3 may directly compete with NF-AT for DNA binding (15). Alternatively, recent work demonstrating the ability of other Foxp subfamily members (Foxp1 and Foxp2) to bind to the transcriptional corepressor CtBP1 (20) raises the possibility that

FOXP3 may inhibit NF-AT activity independent of NF-AT binding, through recruitment of transcriptional corepressors to the promoters of NF-AT-regulated target genes, thereby inhibiting the transcriptional machinery. The identification in this study of a region of FOXP3 outside of the FKH domain that mediates gene repression supports the latter hypothesis as the mechanism of FOXP3-mediated suppression of gene transcription.

To test our hypotheses, the murine T cell line EL4 was cotransfected with various Gal4-FOXP3 fusion constructs and a Gal4/NF-AT luciferase reporter plasmid bearing Gal4 DNA binding sites upstream of NF-AT binding sites (see *Materials and Methods* for details). Cells were stimulated with PMA and ionomycin to activate NF-AT and the extent of suppression of NF-AT-mediated gene expression evaluated for each fragment of FOXP3. Expression of the Gal4- Δ FKH fusion construct resulted in \sim 80% inhibition of activation-induced transcriptional activity in comparison with the Gal4 DBD by itself (Fig. 5A). This is similar to the level of transcriptional repression observed with the Gal4 luciferase reporter using the same fragment of FOXP3 (Fig. 4A). Because EL4 cells do not express endogenous FOXP3 (data not shown) and the transfected FOXP3 construct only bears the DBD of Gal4, these data suggest that FOXP3 does not need to bind to the same site as NF-AT to exert its suppressive effect on NF-AT *trans* activation.

To determine which domains of FOXP3 are required for suppression of inducible NF-AT-mediated gene transcription, the panel of Gal4-FOXP3 fusion constructs used to study FOXP3 activity on the constitutively active SV40 promoter was used. The fusion constructs were cotransfected with the Gal4/NF-AT luciferase reporter, and activation-induced activity was assessed. Expression of a fusion protein containing aa 1–198 of FOXP3 resulted in the same degree of inhibition as the Δ FKH construct (\sim 80%), whereas the ZnF/Zip fragment of FOXP3 had no suppressive activity (Fig. 5A). These data demonstrate that similar to a constitutively active promoter, the domain of FOXP3 required

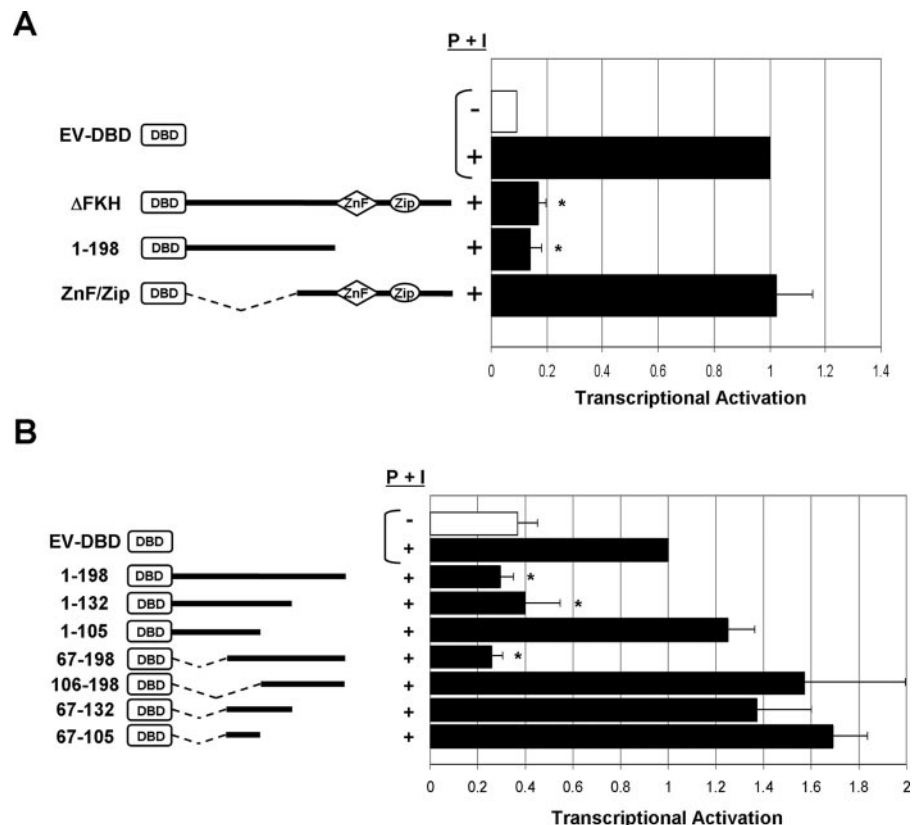
for repression of inducible NF-AT-mediated transcription lies within the N-terminal portion of FOXP3.

As before, the active repressor domain in this system was further defined by using overlapping fragments derived from the first 198 aa of FOXP3 cotransfected with the Gal4/NF-AT luciferase reporter. Overlapping portions of this domain (1–132 and 67–198) both cause significant repression of inducible transcription, but the 67–198 fragment is somewhat more potent (\sim 75 vs 60%) (Fig. 5B). Smaller fragments of this region were again used to focus on the most relevant domain(s). As before, Gal4-FOXP3 fusion proteins bearing aa 106–198 and 67–105 of FOXP3 showed no repressor activity when tested for their ability to repress inducible NF-AT-mediated transcriptional activity. Surprisingly, despite the repressor activity noted previously on a constitutively active promoter, Gal4-FOXP3 fusion proteins bearing aa 1–105 or 67–132 of FOXP3 did not show significant repression of inducible NF-AT transcriptional activity (Fig. 5B). The construct encoding aa 67–198 was, however, fully functional as a repressor in this assay, suggesting that the suppression of inducible NF-AT-mediated transcription may be mechanistically different from the suppression of a constitutively active promoter.

IPEX mutations in FOXP3 result in loss of transcriptional repression

In vitro analysis of FOXP3 activity is very informative as to the function of each structural domain of the protein; however, the ultimate proof of functionality is by analysis in vivo. As previously mentioned, a number of mutations in *FOXP3* have been identified in individuals with IPEX syndrome. These affect three regions of the FOXP3 protein: the FKH DBD, the leucine zipper, and an area in the N-terminal region, which we show to retain the *trans*-repressor function of FOXP3 (Fig. 1) (8, 16–18) (T. Torgerson and H. Ochs, manuscript in preparation).

FIGURE 5. A slightly larger N-terminal domain of FOXP3 is required to repress inducible NF-AT-mediated gene transcription. *A* and *B*, Expression constructs encoding truncation mutants of FOXP3 as shown were cotransfected into EL4 cells with the 5 \times GAL4/NF-AT luciferase and β -galactosidase reporters. Eighteen hours posttransfection, 1×10^6 cells were stimulated with 25 ng/ml PMA and 1.5 μ M ionomycin (P + I) for 6 h, washed in 1 \times PBS, and lysed. Luciferase assays were performed, and luciferase activity was normalized by β -galactosidase activity. The data are an average of three experiments, and the error bars represent the SD from the mean. *, Indicates reduction in activity relative to empty vector is statistically significant ($p < 0.05$).



To validate the functional effect of missense mutations identified in IPEX patients on the transcriptional repressor function of FOXP3, we have generated expression vectors encoding a number of the identified mutant FOXP3 proteins. These were cotransfected

into Jurkat T cells with an IL-2 luciferase reporter vector containing 715 bp of the human *IL-2* promoter (see *Materials and Methods* for details). Transfection of wt FOXP3 results in a marked, dose-dependent reduction in luciferase activity compared with the

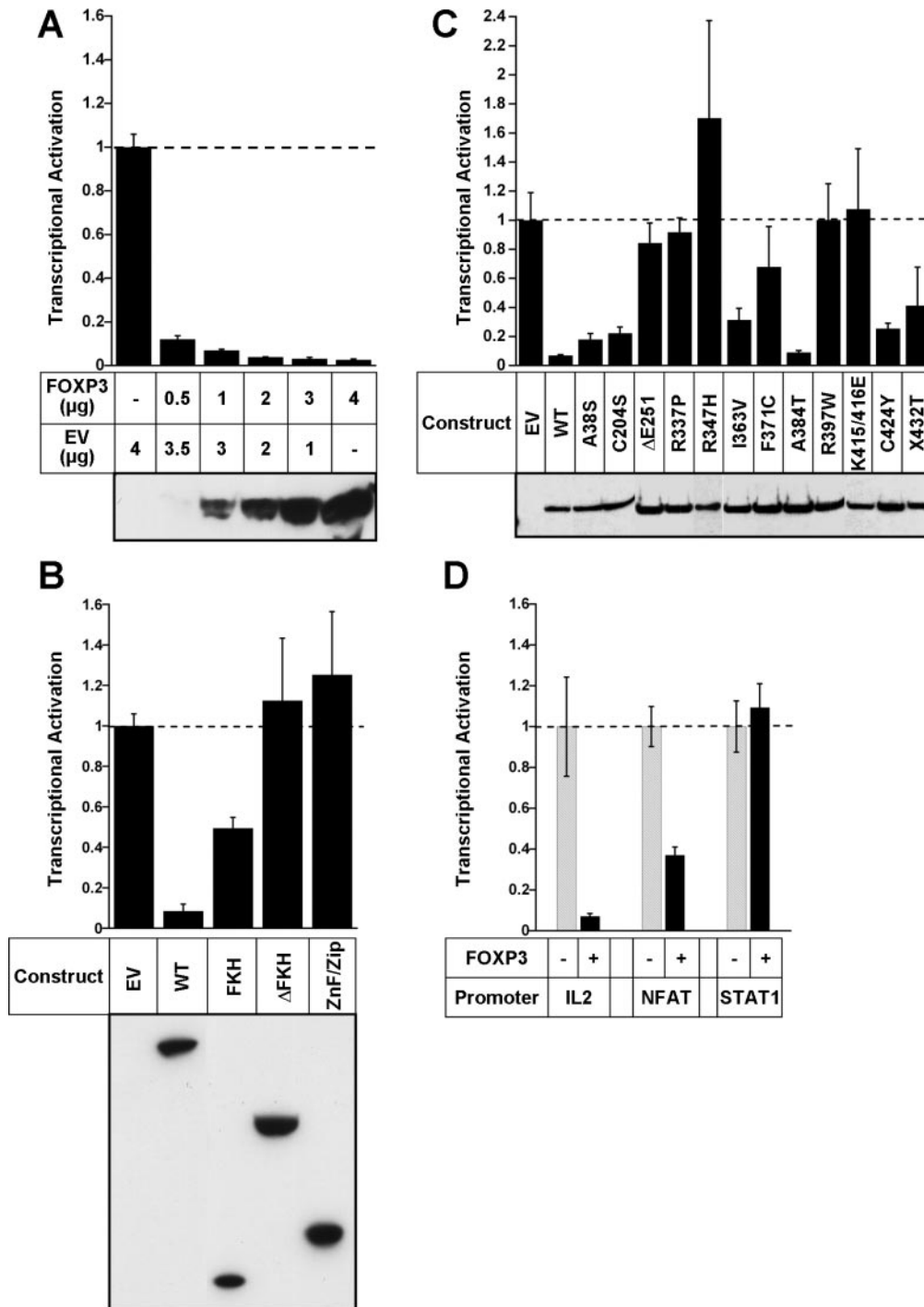


FIGURE 6. Mutations identified in IPEX patients exhibit a loss of repressor activity and demonstrate the in vivo significance of identified structural domains. Expression constructs encoding wt or mutant FOXP3 were cotransfected with a firefly luciferase reporter containing the human *IL-2* promoter and a control *Renilla* luciferase reporter into Jurkat T cells. Eighteen hours posttransfection, cells were stimulated with PMA and ionomycin for 16 h. Luciferase assays were performed using a dual luciferase kit, and all samples were normalized to *Renilla* luciferase activity. The average fold induction in the absence of FOXP3 expression (empty vector (EV)) was set to 1, and all other samples are reported relative to this. The data are an average of at least three experiments, and the error bars represent the SD from the mean. *A*, The wt FOXP3 causes potent and dose-dependent repression of the *IL-2* promoter. *B*, Deletion of particular domains of FOXP3 leads to partial or complete loss of transcriptional repression activity. *C*, Various point mutations identified in IPEX patients differentially affect transcriptional repression activity of FOXP3. *D*, Transcriptional repression by FOXP3 potently affects NF-AT-mediated gene transcription (*IL-2* and *NF-AT*), but is not the result of global suppression, as demonstrated by its lack of effect on IFN- γ -mediated induction of *STAT1* transcriptional activity. Western blots shown below each graph demonstrate the expression of each FOXP3 construct.

empty vector control (Fig. 6A). Transcriptional repression is restricted to promoters dependent on NF-AT for induction because constructs bearing either the human IL-2 promoter or three tandem NF-AT binding sites are repressed, whereas activation of a promoter consisting of three tandem STAT1 binding sites is unaffected (Fig. 6D).

Mutant FOXP3 proteins analogous to the *scurfy* mutation (Δ FKH) or consisting only of the central region of the protein, including the leucine zipper and zinc finger (ZnF/Zip), showed a complete lack of repressor activity (Fig. 6B), suggesting that nuclear import and/or DNA binding are necessary for this effect. A mutant encoding only the FKH domain is capable of nuclear import (Fig. 2) and exhibited partial (~50%) repressor activity, confirming that this domain plays a role in suppression, but requires the presence of the leucine zipper and N-terminal region for full repressor function.

Similar to the Δ FKH mutant lacking the FKH domain, the two point mutations that affect nuclear import of FOXP3 (K415/416E and R347H) have markedly diminished repressor activity compared with the wt protein (Fig. 6C). The R347H mutation may actually enhance transcription somewhat, although the increased promoter activity was not statistically significant.

The leucine zipper mutant, found to affect homodimerization (Δ E251; Fig. 3), exhibits markedly reduced repressor activity on the IL-2 promoter, suggesting the importance of dimerization either for DNA binding or for the repressive function of FOXP3 (Fig. 6C). This is a reasonable explanation, because it has been demonstrated that homodimerization of Foxp1, Foxp2, and Foxp4 is required for DNA binding and repression in the model system that was tested (20). A mutation that abrogates the structure of the zinc finger (C204S) has only mildly diminished repressive activity compared with wt FOXP3, supporting the assertion that the zinc finger does not play a significant role in the repressor function of FOXP3. This may explain why no mutations in the zinc finger domain have been identified in patients with IPEX. There remains the possibility, however, that the canonical C2H2 zinc finger affects an aspect of FOXP3 function that has not yet been identified.

Only one missense mutation in the N-terminal region was tested (A38S), and this lies outside of the repressor domain that we have identified. This mutant exhibited almost full repressive function in the assay, suggesting either that this mutation is not actually the cause of IPEX in these patients or that it affects another aspect of FOXP3 function. It does not appear to affect protein stability when overexpressed in HEK 293 or Jurkat cells (Fig. 6C and data not shown).

The remaining missense mutations tested in this assay were identified in the FKH DBD of FOXP3 in patients with IPEX, and all but the A384T mutation within this region led to some reduction in repressor function (Fig. 6C). Interestingly, the A384T mutation has been identified in three families with affected boys who have the classical features of IPEX syndrome. One of the affected males has been evaluated by flow cytometry for the presence of CD4⁺CD25⁺FOXP3⁺ T_{reg}s, and these were found to be absent, arguing that this mutation is not simply a polymorphism and suggesting that it may alter another function of FOXP3 (T. Torgerson and H. Ochs, manuscript in preparation). It is possible that this mutation affects the stability of mRNA or protein in cells of the affected individual. In this experimental system, in which the transfection of plasmids results in FOXP3 expression at superphysiologic levels, it is possible that subtle differences in repressor function may be missed or minimized. These possibilities are being investigated. The decreased repressor function exhibited by most of the missense mutations in the FKH domain does, however, sug-

gest that the ability of the FKH domain to mediate DNA binding is critical to the function of FOXP3.

Collectively, these data argue that the molecular basis of IPEX in these individuals is the result of an inability to regulate transcription of target genes, ultimately leading to a lack of T_{reg} development and function.

Discussion

FOXP3 has been identified as a critical regulator of T_{reg} development and function (10–14). Because of its homology to the FKH family of transcription factors, it is presumed that FOXP3 mediates its effects through transcriptional regulation of specific target genes.

Our previous studies have shown that, when expressed in cell lines, FOXP3 localizes to the nucleus and functions as a repressor of transcription from both a constitutively active promoter (SV40) and a promoter that is inducibly activated by the transcription factor NF-AT (IL-2). Both its nuclear localization and its repressor functions were found to be dependent on the presence of the FKH domain, because a mutant protein lacking the FKH domain did not localize to the nucleus and did not repress transcription (15). We also showed that FOXP3 binds to DNA containing a consensus FKH domain-binding sequence, and this DNA-binding ability is dependent on its FKH domain. We therefore set out to define the regions of FOXP3 that possess critical functional activities to gain a better understanding of the mechanism by which FOXP3 represses transcription of its targets. Using luciferase reporter plasmids containing binding sites for the yeast Gal4 DBD and expression constructs encoding Gal4-FOXP3 fusion proteins, we show that FOXP3 contains at least two domains required for repressor function, and that these domains lie within the first 198 aa of FOXP3. The domain required for repression of the constitutively active SV40 promoter was localized to a region between aa 67 and 132. This pattern mirrors the observation made using the same assay system, that in Foxp subfamily members Foxp1 and Foxp2, a repressor domain lies within the central region of the protein, adjacent to the zinc finger and leucine zipper (20). In Foxp1 and Foxp2, this domain mediates a protein-protein interaction with CtBP1, a transcriptional corepressor known to bind to members of class II histone deacetylases (20, 27). Neither FOXP3 nor Foxp4 contains the required binding domain for CtBP1 (20). We hypothesize that a sequence within aa 67–132 of FOXP3 also mediates a protein-protein interaction with a transcriptional corepressor, most likely different from CtBP1.

The finding that a Gal4-FOXP3 fusion protein containing the first 198 aa of FOXP3 represses activation-induced NF-AT-mediated transcriptional activity when separate binding sites for Gal4 are placed upstream of NF-AT binding sites suggests that FOXP3 exerts its effect through a *trans*-repression mechanism and not by direct competition for sites within, or adjacent to, NF-AT binding sites. Interestingly, the Gal4-FOXP3 fusion protein consisting of aa 67–132 is not sufficient to repress NF-AT-mediated transcriptional activity. One explanation is that EL4 cells lack a cofactor that is present in HEK 293 T cells, which interacts with aa 67–132. This is unlikely because wt FOXP3 is able to repress transcription of the same reporter in EL4 cells. Another, more plausible explanation is that an additional repressor domain, also localized to the N-terminal region of FOXP3, is required to repress NF-AT-mediated transcriptional activity. This second repressor domain may mediate a direct interaction with NF-AT. This notion is supported by a recent study of murine Foxp3, demonstrating its association with NF- κ B and NF-AT (28). Using human cells and proteins, we have been unable to demonstrate a direct interaction between FOXP3 and NF-AT2 by coimmunoprecipitation even when both

are simultaneously overexpressed (data not shown). It is possible that the interaction between the two proteins is of low affinity and that it requires that the complex be assembled on DNA to improve stability. Attempts to coimmunoprecipitate FOXP3 and NF-AT2 have, however, been unsuccessful even in the presence of double-stranded oligonucleotides bearing typical NF-AT binding sites (data not shown). In this same report, Foxp3 was shown to inhibit activation-induced IL-2, IL-4, and IFN- γ production in retrovirally transduced primary T cells. Furthermore, the first 199 aa of murine Foxp3 were shown to be required for the inhibition of IL-2 production (28).

Guided by mutations identified in patients with IPEX, we have also investigated other vital functions of FOXP3, including homodimerization and nuclear import. Foxp1, Foxp2, and Foxp4 can form homo- and heterodimers with each other, and their dimerization as well as their transcriptional repressor function are dependent on their leucine zipper domains (20). We show in this study that FOXP3 forms homodimers, and its dimerization is dependent on the leucine zipper, because deletion of a conserved glutamic acid residue (Δ E251) in the leucine zipper inhibits dimerization (Fig. 3). In addition, this mutation abrogated the repressor function of FOXP3 (Fig. 6C). In contrast, a mutation that destroys the structure of the zinc finger did not affect dimerization or repressor function (Figs. 3 and 6C). The physiologic pertinence of these findings is suggested by the fact that four unrelated patient families with IPEX have been identified with the Δ E251 mutation and two other families identified with other mutations in the leucine zipper (T. Torgerson and H. Ochs, manuscript in preparation); however, no mutations have been identified in the zinc finger domain.

We identify two regions that are involved in nuclear localization and demonstrate that nuclear localization of FOXP3 is required for transcriptional repression. A putative NLS at the carboxyl end of the FKH domain appears to act as the primary targeting sequence, but a point mutation at the amino end of the FKH domain (R347H, identified in a patient with IPEX) has a partial effect on nuclear targeting (Fig. 2) and completely abrogates repression of IL-2 transcription (Fig. 6C). The involvement of sequences at both ends of the FKH domain has been observed in at least one other Fox family member (22). It is possible that instead of limiting nuclear import, this mutation creates a nuclear export signal; however, this possibility is difficult to differentiate experimentally from inhibition of nuclear import.

As shown in Fig. 1, mutations have been identified along the length of FOXP3 in patients with the fatal autoimmune disease IPEX. By evaluating transcriptional repressor activity, we are the first to demonstrate a functional consequence for mutations of FOXP3 analogous to those found in IPEX. As shown previously, loss of the FKH domain altogether results in loss of repressor function (15) (Fig. 6B). Demonstration in this study, that point mutations within the FKH domain also lead to loss of function, further argues that the ability of FOXP3 to bind to DNA is critical to its repressor activity. Likewise, mutation of a conserved glutamic acid residue within the leucine zipper (Δ E251), which inhibits homodimerization of FOXP3, also reduces the repressor activity of FOXP3. This result demonstrates that homodimerization of FOXP3 is essential for FOXP3 function in vivo.

Identification of IPEX-associated mutations in and around specific functional domains, and the identification of precise defects in molecular function resulting from specific mutations provide the definitive, causative link between FOXP3 dysfunction and IPEX in humans. This link has also been clearly demonstrated in mice, in which mutation or deletion of Foxp3 results in a fatal autoimmune disease characterized by dysregulated proinflammatory cytokine production, multiorgan lymphocytic infiltrates, lymphadenopathy,

splenomegaly, and the production of autoantibodies (5, 12, 13). Several groups have outlined the requirement of Foxp3 for the development of T_{regs} in mice, and recently flow cytometric studies in IPEX patients showing absence of T_{regs} suggested that functional FOXP3 is required for the development of this important T cell population in humans (10–14). It is known that transduction of *FOXP3* into human primary T cells (23) and *Foxp3* into mouse primary T cells results in the T cells acquiring a T_{reg}-like phenotype with reduced IL-2 and IL-10 production in response to TCR ligation and up-regulation of T_{reg}-associated cell surface Ags (10–12, 29). These data suggest that FOXP3 alters the genetic program of normal T cells to generate the regulatory phenotype and that dysregulated expression of the transcriptional targets of FOXP3 in IPEX and *Scurfy* underpins the abnormal development and function of T_{regs}, thus leading to severe autoimmunity. The identification of these transcriptional targets will be pivotal to understanding the ontogeny and function of naturally arising T_{regs}.

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