Transcriptional Regulation by Foxp3 Is Associated with Direct Promoter Occupancy and Modulation of Histone Acetylation*§

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Regulatory T cells (Treg), a forkhead family member that is necessary and sufficient for Treg lineage choice and function. Ectopic expression of Foxp3 in non-Treg leads to repression of the interleukin 2 (IL-2) and interferon γ (IFNγ) genes, gain of suppressor function, and induction of genes such as CD25, GITR, and CTLA-4, but the mode by which Foxp3 enforces this program is unclear. Using chromatin immunoprecipitation, we have demonstrated that Foxp3 binds to the endogenous IL-2 and IFNγ loci in T cells, but only after T cell receptor stimulation. This activation-induced Foxp3 binding was abrogated by cyclosporin A, suggesting a role for the phosphatase calcineurin in Foxp3 function. We have also shown that binding of Foxp3 to the IL-2 and IFNγ genes induces active deacetylation of histone H3, a process that inhibits chromatin remodeling and opposes gene transcription. Conversely, binding of Foxp3 to the GITR, CD25, and CTLA-4 genes results in increased histone acetylation. These data indicate that Foxp3 may regulate transcription through direct chromatin remodeling and show that Foxp3 function is influenced by signals from the TCR.

Acquired immune tolerance depends upon a subset of T cells that suppress the differentiation and function of pathogenic effector T cells. These regulatory T cells (Treg) specifically express Foxp3, a member of the forkhead family of DNA-binding proteins that appears to be necessary and sufficient for Treg lineage choice (1–3). Humans with mutations in the foxp3 gene suffer from an X-linked complex of immune dysregulation, polyendocrinopathy, and enteropathy, a syndrome that results in part from the lack of Treg and leads to eventual death of these patients in childhood (4). Similarly, Treg are crucial for the inhibition of immunopathology in experimental models of organ transplantation and early onset autoimmune disorders and have been implicated in the control of childhood (type 1) autoimmune diabetes mellitus (5, 6).

Recent studies have shown that Foxp3 overexpressed in transformed cell lines can repress transcription from artificial forkhead, nuclear factor of activated T cell (NFAT),3 or NFκB consensus binding elements in transiently transfected plasmids (7–9). These data suggest that Foxp3, like Foxp1 and Foxp2 (10), can function as a transcriptional repressor. However, expression of Foxp3 also leads to the induction of multiple genes, but the molecular basis for this activity is not known. It is also clear that Foxp3 expression alone is not sufficient for regulatory activity, as Foxp3+ Treg must receive antigenic signaling to exert suppressor function (11), but the basis for this activation requirement is not clear.

We have used chromatin immunoprecipitation (ChIP) to analyze binding and remodeling of the chromatin at several Foxp3-responsive genes. Our studies provide important insight into how Foxp3 may regulate anergy in Treg and suggest a potential explanation for why Treg require T cell receptor (TCR) activation for their suppressive functions.

EXPERIMENTAL PROCEDURES

Cells—A Jurkat human T cell line expressing the ecotropic retroviral receptor (mCAT-1) (12) was used for these studies. CD4+ CD25− and CD4+ CD25+ were purified from C57BL/6 mice using a Treg isolation kit (Miltenyi). Treg were also induced in vitro by culturing CD4+ CD25− for 3–4 days on plate-bound anti-CD3 in the presence of soluble anti-CD28 (0.5 μg/ml each) and TGFB (5 ng/ml).

Retroviral Transduction—Murine Foxp3 cDNA was amplified from C57BL/6 thymus and cloned into the murine stem cell virus (MSCV)-based MIGR1 and MINR1 retroviral vectors (13), and versions of each vector were constructed containing an in-frame, N-terminal FLAG epitope. For generation of retrovirus, constructs were cotransfected with the pCLeco (Invitrogen) helper plasmid into the 293T-based Phoenix ecotropic packaging cell line (provided by G. Nolan, Stanford University). CD4+ CD25− T cells were activated with phosphor 12-myristate 13-acetate (3 ng/ml), ionomycin (1 μM), and IL-2 (10 units/ml) for 24 h, washed, and transduced by spinfection.

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3 The abbreviations used are: NFAT, nuclear factor of activated T cell; ChiP, chromatin immunoprecipitation; TGFB, transforming growth factor β; IL, interleukin; qRT-PCR, quantitative reverse transcription PCR; Ab, antibody; IFNγ, interferon γ; CsA, cyclosporine A; NGFR, nerve growth factor receptor; GITR, glucocorticoid-induced TNF receptor-related protein.
(13) with 24–48 h Phoenix supernatants. Transduced cells were expanded in IL-2 for 1–3 days. The mCAT-1 Jurkat line was transduced as above with no activation. The MIGR1-transduced Jurkat cells depicted in Figs. 2–4 were purified by fluorescence-activated cell sorter and maintained as stable lines. The transduced CD4+ T cells depicted in Figs. 1 and 2 were enriched to 92–97% purity before analysis using anti-NGFR-coupled magnetic beads. In the experiments depicted in Figs. 3–5, CD4+ T cell transduction efficiencies were >80%.

**qRT-PCR, Immunoblot, and Immunocytochemical Analysis**—Total RNA was extracted from 1–2 × 10^6 cells and amplified using primer/probe sets (Applied Biosystems) against GITR, CTLA-4, TGFβ, IL-2, and 18 S RNA. Whole cell protein extracts were prepared in Laemml buffer, and cytoplasmic and nuclear extracts were prepared using a commercial kit (Pierce). For immunoblot analysis, extracts of 0.5–1 × 10^6 cells were subjected to SDS-PAGE, blotted to nitrocellulose, blocked, and probed with Ab against the FLAG epitope (Sigma), native Foxp3 (catalogue numbers 14–5773 and 14–7979; eBio-science), Sp1 (Santa Cruz Biotechnology), or actin (Sigma). For immunocytochemistry, cytospun cells were fixed in periodate-lysine-parafomaldehyde and permeabilized using Triton X-100 (0.1%); Foxp3 was localized by overnight incubation with anti-Foxp3 monoclonal antibody (catalogue number 14–5773; eBioscience) followed by rabbit anti-mouse IgG and developed using an Envision immunoperoxidase kit (Dako).

**In Vitro Suppression Assay**—Purified CD4+CD25+ Treg or transduced CD4+ T cells were added at the indicated ratios (Treg:target) to a 1:3 mixture of naïve, carboxyfluorescein succinimidyl ester-labeled CD4+CD25− target T cells and T cell-depleted, irradiated splenocytes. Cells were cultured with soluble anti-CD3 (0.5 μg/ml) for 3 days.

**Chromatin Immunoprecipitation**—Chromatin-DNA complexes were prepared from 1–2 × 10^6 cells and subjected to quantitative ChIP analysis as described previously (14), using 10 μg of antibody against either acetylated histone H3 (Upstate), FLAG epitope (Sigma), or native Foxp3. Control precipitations using nonspecific antibody were also performed from the same chromatin extracts to establish the nonspecific background. Genomic DNA extracted from each precipitate was probed by qRT-PCR for the regulatory regions of the IL-2 (14), IFNγ (15), GITR, CTLA-4, and CD25 genes (see supplemental Table S1 for primer sequences).

**RESULTS**

**Ectopic Expression and Function of Foxp3 in CD4+ T Cells**—To study the basis of gene regulation by Foxp3, we utilized an ectopic model in which Foxp3 is expressed via retroviral transduction in primary, non-regulatory lineage CD4+ T cells (1). Murine stem cell virus-based retroviral constructs (13) (Fig. 1A) carrying green fluorescent protein (Fig. 1B) or mutant human nerve growth factor receptor (NGFR) (Fig. 1C) reporters were used to express wild-type murine Foxp3 or an N-terminal FLAG-tagged version of Foxp3 in primary CD4+CD25− T cells. Foxp3-transduced CD4+ T cells, but not control empty vector-transduced cells, exhibit specific expression of both Foxp3 mRNA (Fig. 1D) and protein (Fig. 1E) at levels roughly 2-fold higher than natural CD4+CD25+ Treg. Natural CD25+ Treg constitutively express GITR, CTLA-4, and TGFβ (Ref. 11 and Fig. 2, A–C, left panels), and consistent with previous studies (1, 7, 8, 16, 17) we found that ectopic expression of Foxp3 in CD4+ T cells recapitulates this genetic program (Fig. 2, A–C, right panels). We also confirmed that, like natural CD25+ Treg, Foxp3-transduced CD4+ T cells could suppress the proliferation of naïve CD4+ T cells (Fig. 2D). This gain of function was Foxp3 specific, as empty vector-transduced CD4+ T cells could not suppress in the same assay (Fig. 2D). We also confirmed that, like natural CD25+ Treg (Fig. 2E), Foxp3-transduced T cells were defective in activation-induced IL-2 gene expression at the level of both mRNA and protein (Fig. 2, F and G). This defect in IL-2 production was observed with both wild-type and FLAG-tagged Foxp3 constructs (data not shown). The Foxp3-transduced T cells also failed to produce IFNγ in response to TCR ligation (Fig. 2H). These data validate this ectopic expres-
whether Foxp3 binds to the endogenous forkhead family members (9, 18, 19). To determine ester-labeled target CD4 at putative binding elements on both DNA strands, is located to the endogenous IFN showed specific binding of Foxp3 to the proximal and distal but not with empty vector, using antibodies specific for either murine CD4/CD25, CD4/CD25, and CD4/CD25, or MIGR1-Foxp3 transduced Jurkat cells (G) were stimulated for 8 h with anti-CD3 Ab, and IL-2 (E and F) and IFNγ (E) mRNA and protein (ng/ml) were assessed. Values for GTP, CTLA-4, TGFβ, IFNγ, and IL-2 mRNA were normalized to 18 S RNA and are graphed as a -fold increase over the control-transduced or the CD4+CD25+ values. Results are representative of at least three separate experiments.

Foxp3 Binds to the Endogenous IL-2 and IFNγ Loci in T Cells in a TCR-dependent Manner—We identified six forkhead transcription factor consensus elements in the region 1 kb upstream of the murine IL-2 locus (Fig. 3A) and five elements in the defined promoter and enhancer regions of the IFNγ locus (Fig. 3B), suggesting that Foxp3 may bind to the regulatory region of these genes and act as a direct transcriptional repressor. One of these sites in the IL-2 promoter/enhancer, which contains putative binding elements on both DNA strands, is located adjacent to the ARRE-2 NFAT/AP-1/Oct-1 response element at ~263 bp (Fig. 3A, asterisk) and has been shown to bind several forkhead family members (9, 18, 19). To determine whether Foxp3 binds to the endogenous IL-2 and IFNγ genes, we employed chromatin ChIP analysis. We were able to detect in vivo binding of Foxp3 to the endogenous IL-2 promoter (Fig. 3C) and the endogenous IFNγ intronic enhancer (Fig. 3D) in murine CD4+ T cells transduced with FLAG-tagged Foxp3, but not with empty vector, using antibodies specific for either FLAG or Foxp3. Similarly, Foxp3-transduced Jurkat T cells showed specific binding of Foxp3 to the proximal and distal regions upstream of the IL-2 gene (Fig. 3, E and F) as well as to the IFNγ promoter (Fig. 3G).

We were also able to detect binding of native Foxp3 protein to the endogenous IL-2 promoter in Foxp3+ Treg induced in vitro by TGFβ, but not in Foxp3− CD4+ T cells (supplemental Fig. S1). These results demonstrate that Foxp3 promoter occupancy is a physiologic phenomenon in natural Treg and not merely an artifact of ectopic expression.

Interestingly, Foxp3 binding to the regulatory elements of these cytokine genes was highly dependent upon active TCR/CD28 stimulation. Foxp3-transduced CD4+ T cells that were allowed to come to rest after activation exhibited little or no Foxp3 promoter occupancy at the IL-2 promoter (Fig. 3H), the IFNγ promoter (Fig. 3J), or the IFNγ intronic enhancer (Fig. 3J), whereas restimulation of Foxp3-transduced T cells through the TCR and CD28 restored strong, specific Foxp3 binding at all these regions (Fig. 3, H–J). This activation-induced Foxp3 binding could be simulated by the combination of diacylglycerol analog and Ca2+ ionophore (Fig. 3K), implicating protein kinase C, RasGRP, and/or Ca2+ signaling in promoting Foxp3 chromatin binding. Consistent with this interpretation, inhibition of Ca2+-dependent calcineurin phosphatase activity during T cell activation using cyclosporine A (CsA) abrogated Foxp3 binding to the IL-2 promoter (Fig. 3L) and the IFNγ enhancer (Fig. 3M). These data demonstrate that TCR-coupled Ca2+ signaling through calcineurin is required for Foxp3 binding to chromatin at the IL-2 and IFNγ genes and provides a molecular framework for why Treg suppressor function requires activation and is CsA sensitive (11).

TCR Signaling Influences the Subcellular Localization of Foxp3—The data in Fig. 3 show that Foxp3 promoter occupancy at inflammatory cytokine genes is dependent upon signals from the antigen receptor and suggest that Foxp3 function may be modulated post-translationally by TCR signaling. Consistent with this, we found that activation through TCR/CD28 induces a shift in the subcellular localization of Foxp3 from a primarily cytoplasmic/perinuclear pattern in most cells to a dense nuclear pattern in both natural CD4+CD25+ Treg and Foxp3-transduced CD4+ T cells (supplemental Fig. S2, A–D). This was confirmed biochemically in Foxp3-transduced Jurkat T cells in which the ratio of Foxp3 present in nuclear versus cytoplasmic extracts was increased after TCR ligation (supple-
mental Fig. S2E). This shift in the subcellular localization of Foxp3 was not significantly affected by CsA (data not shown), suggesting that the requirement for TCR signals for Foxp3 promoter occupancy is not at the level of nuclear localization and implying that other TCR-coupled signaling pathways must be involved in regulating the subcellular distribution of this protein.

**Foxp3 Induces Histone Deacetylation at the IL-2 and IFNγ Loci**—Transcriptional regulators commonly function by modulating the local chromatin structure at gene promoters and enhancers. An initial step in the relaxation of chromatin structure is the enzymatic addition of acetyl groups to the positively charged tails of histone H3 and H4 by histone acetyltransferases (20). Histone acetylation often accompanies gene transcription...
Chromatin Remodeling by Foxp3

**FIGURE 4. Effect of Foxp3 binding on ACh3 at the IL-2 and IFNγ loci.** CD4+ T cells (A–C) or Jurkat cells (D–F) transduced with MIGR1 empty vector (light gray bars) or MIGR1-FLAG-Foxp3 (dark gray bars) were cultured as indicated and subjected to ChIP analysis using ACh3 Ab or with control nonspecific Ab. Precipitated genomic DNA was probed for the promoter/enhancer regions of the IL-2 (A, B, D, and E) or IFNγ (C and F) loci by qRT-PCR. Histone acetylation was calculated as the anti-ACh3 ChIP signal divided by the nonspecific Ab background ChIP. Asterisk in panel B denotes undetectable ACh3. Results depicted are representative of three to five separate experiments.

IL-2 promoter (Fig. 4B, dark gray bars), and secondary recruitment of Foxp3 to the IL-2 promoter following restimulation resulted in a further 30-fold decrease in ACh3 to undetectable levels (Fig. 4B, dark gray bars). Foxp3 also induced activation-dependent histone deacetylation at the IFNγ enhancer in transduced CD4+ T cells (Fig. 4C, dark gray bars). Jurkat T cells transduced with empty MIGR1 vector likewise exhibited activation-induced ACh3 upstream of the IL-2 and IFNγ genes (Fig. 4, D–F, light gray bars). However, expression of Foxp3 in these cells resulted in marked histone deacetylation at these same regions (Fig. 4, D–F, dark gray bars). Together, these data show that Foxp3 binding to the IL-2 and IFNγ loci in T cells induces local histone deacetylation and suggest that the transcriptional effects of Foxp3 may function through recruitment of histone deacetylases and modulation of chromatin structure.

Foxp3 Increases Histone Acetylation at the GITR, CD25, and CTLA-4 Loci—Our data above show that Foxp3 directly binds to the IL-2 and IFNγ loci and induces strong deacetylation of the histones in these regions, which is consistent with the behavior of a transcriptional repressor. However, expression of Foxp3 in T cells also leads to the induction of genes such as GITR, CD25, and CTLA-4. If Foxp3 is only capable of transcriptional repression, then Foxp3 must regulate these genes indirectly by repressing genes that normally negatively regulate GITR, CD25, and CTLA-4 expression. Alternatively, if Foxp3 is capable of functioning as either a repressor or an activator then one may predict that Foxp3 would bind directly to all these genes but regulate chromatin structure differently in the context of the repressed versus the induced loci.

To test this, we measured Foxp3 binding and ACh3 at the GITR, CD25, and CTLA-4 promoters in control- and Foxp3-transduced CD4+ T cells. We were able to detect specific binding of Foxp3 to the promoter regions of GITR, CD25, and CTLA-4 (Fig. 5, A–C), suggesting that Foxp3 could potentially regulate these genes directly. Whereas Foxp3 binding to the IL-2 and IFNγ loci led to histone deacetylation, binding of Foxp3 to the GITR, CD25, and CTLA-4 promoters conversely resulted in increased histone acetylation at these regions (Fig. 5, D–F). This modification of the chromatin structure generally facilitates gene transcription and is consistent with Foxp3 serving as a direct activator of CD25, GITR, and CTLA-4 gene expression.

**DISCUSSION**

Our studies here have demonstrated that Foxp3 binds directly to the promoter and enhancer regions of the endogenous IL-2 and IFNγ genes in T cells. This binding is associated with marked histone deacetylation and repression of gene transcription. Our studies also demonstrated that the binding of Foxp3 to these loci is absolutely dependent upon T cell activation and suggest a scenario in which the TCR-CD28 complex transduces signals that influence Foxp3 function.

This regulation could be mediated directly by TCR-mediated post-translational modifications that influence the subcellular distribution or DNA binding affinity of Foxp3. Indeed, several other forkhead transcription factors (e.g. Foxo1–4, Foxm1, Foxa2) are known to be regulated by site-specific phosphorylation (28–30), and the primary structure of Foxp3 suggests that

(21), is required for the appropriate tissue-specific and context-dependent induction of many genes (22–24), and is opposed by the activity of histone deacetylases (25). To determine whether Foxp3 might regulate IL-2 and IFNγ gene expression through chromatin remodeling, we measured histone acetylation (ACh3) at the IL-2 and IFNγ loci in activated, Foxp3-expressing T cells. The chromatin at the IL-2 and IFNγ promoters in quiescent, naïve CD4+ T cells exists in a "closed" state (14, 26, 27), and the histones in this region are not acetylated (Ref. 14 and Fig. 4A). Following productive T cell activation, histones at the IL-2 and IFNγ loci are rapidly and stably acetylated (Ref. 14 and Fig. 4, A–C), and the chromatin at the IL-2 promoter is remodeled to form an accessible structure (14, 26, 27). A significant amount of ACh3 remained at the IL-2 promoter after these activated cells were allowed to come to rest following MIGR1 transduction (Fig. 4B, light gray bars). However, transduction with MIGR1-Foxp3 blocked activation-induced ACh3 at the
this protein contains multiple motifs that may serve as substrates for TCR-coupled protein kinases. Consistent with this, we find that TCR signaling induces a shift in the subcellular pattern of expression of Foxp3. One of the signals involved in activation-induced Foxp3 promoter occupancy is apparently transduced through the Ca\textsuperscript{2+} activation-induced Foxp3 promoter occupancy is apparently transduced through the Ca\textsuperscript{2+} -dependent phosphatase calcineurin, as cyclosporine A treatment completely abrogated Foxp3 binding to the IL-2 promoter in activated T cells (9) and that the IL-2 promoter fails to undergo nucleosome remodeling in these cells (32), our data imply that Foxp3 acts as a direct transcriptional repressor of both the endogenous IL-2 and IFN\textgamma loci.

Conversely, Foxp3 also binds to the endogenous promotor regions of the Foxp3-induced GITR, CD25, and CTLA-4 genes, and this is associated with increased histone acetylation. These results suggest that Foxp3 recruits histone acetyltransferase enzymes, which are powerful co-activators of transcription, to these loci and provide a potential explanation for why Foxp3 induces expression of the GITR, CD25, and CTLA-4 genes while repressing expression of the IL-2 and IFN\textgamma genes. This type of behavior is exhibited by members of the Foxo forkhead subfamily, which have been shown to interact with both histone acetyltransferase and histone deacetylase complexes (33). Indeed, Foxp3 was recently shown to interact with CREB-binding protein in transfected human embryonic kidney 293T cells (34), but whether Foxp3 can recruit this histone acetyltransferase to gene promoters in T cells is not known.

Several important questions remain, including how Foxp3 function is regulated, whether Foxp3 promoter occupancy requires direct DNA binding, which co-repressor or co-activator complexes are recruited by Foxp3 to the promoters of Foxp3-responsive genes, and whether regulation of chromatin structure is necessary for the gene regulation by Foxp3.

Our data offer important insights into how Foxp3 may regulate gene expression and T\textsubscript{reg} function and support a scenario in which Foxp3 is responsive to signals from the TCR and can function either as a direct repressor or activator of transcription. Furthermore, our results imply that the recruitment of histone acetyltransferase-containing co-activator versus histone deacetylase-containing co-repressor complexes may act as a context-dependent biochemical switch that determines the specific regulatory activity of Foxp3 at a given gene.

\textsuperscript{4} C. Chen and A. D. Wells, unpublished observations.
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REFERENCES

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