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Cutting Edge: A Novel, Human-Specific Interacting Protein Couples FOXP3 to a Chromatin-Remodeling Complex That Contains KAP1/TRIM28

Chunjian Huang,* Sunil Martin,† Christian Pfleger,* Jianguang Du,*∥ Jane H. Buckner,‡ Jeffrey A. Bluestone,§ James L. Riley,‡ and Steven F. Ziegler*

Regulatory T cells (Tregs) play a pivotal role in the maintenance of immunological self-tolerance. Deficiency or dysfunction of Tregs leads to severe autoimmune diseases. Although the forkhead/winged-helix family member FOXP3 is critical for Treg differentiation and function, the molecular basis for FOXP3 function remains unclear. In this study, we identified and characterized a human-specific FOXP3-interacting protein, referred to as FIK (FOXP3-interacting KRAB domain–containing protein). FIK is highly expressed in Tregs and acts as a bridging molecule to link FOXP3 with the chromatin-remodeling scaffold protein KAP1 (TIF-1β/TRIM28). Disruption of the FOXP3–FIK–KAP1 complex in Tregs restored expression of FOXP3-target genes and abrogated the suppressor activity of the Tregs. These data demonstrate a critical role for FIK in regulating FOXP3 activity and Treg function. The Journal of Immunology, 2013, 190: 4470–4473.

A n important aspect of immune regulation is the establishment and maintenance of tolerance to self-Ags. In the periphery, this is largely accomplished by a population of CD4 T cells, referred to as regulatory T cells (Tregs), which express the transcription factor Foxp3 (1). These cells are critical for the suppression of pathological immune responses to both self- and foreign Ag, and deficiency or dysfunction of Tregs leads to severe autoimmune disease (2, 3). The development and function of Tregs are dependent on the forkhead/winged-helix transcription factor FOXP3 (4–6); ablation of Foxp3 in mice, as well as FOXP3 mutations in humans with immune dysfunction, polyendocrinopathy, enteropathy, X-linked syndrome, leads to the rapid onset of fatal autoimmune lymphoproliferative disease (7). In addition, ectopic expression of FOXP3 or Foxp3 in naive CD4 T cells from humans and mice, respectively, leads to the generation of T cells with suppressor activity (4, 8). Although the importance of FOXP3 in Tregs is clear, the molecular mechanism by which it controls gene expression in Tregs is largely unknown. In this article we show that, in human Tregs, a novel interacting protein couples FOXP3 to a repressive chromatin-remodeling complex that includes the adaptor KAP1/TRIM28. The interacting protein is found only in human Tregs and demonstrates a fundamental difference in the mechanism by which FOXP3 regulates transcription in human and mouse Tregs.

Materials and Methods

Yeast 2-hybrid screen

The yeast 2-hybrid screen was performed as described previously (9).

Cell culture and transfection

HEK 293T cells and Jurkat T cells were maintained as previously described (9). HEK 293T cells were transfected using TransIT-T1 Transfection Reagent (Mirus, Madison, WI). Jurkat T cells were transfected using Amaxa Cell Line Nucleofector Kit V (Lonza). CD4 T cells were isolated from PBMCs using a human Naive CD4 T Cell Isolation Kit II (Miltenyi Biotec, Auburn, CA). CD4*CD25+ naive T cells and CD4*CD25+ Tregs were isolated by cell sorting.

Reverse transcription and quantitative PCR

Total RNA was isolated using TRIzol reagent and GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO). Quantitative real-time PCR (qPCR) was performed, as described (9), using the following primers: human FOXP3, forward: 5′-TGCCAAGGCTCTCATCTGTG-3′ and reverse: 5′-GAGGAACTCTGGAGATGTC-3′; human IFN-γ, forward: 5′-GGTTCAGACAGCAGGAGGTGA-3′ and reverse: 5′-GCCAGAAGGTCAG- CAGACGC-3′; human IL-2, forward: 5′-ATTACAAGATCACAAACT-C-3′ and reverse: 5′-ATTGCTGATATTAGTCCC-3′; human GAPDH, forward: 5′-ACCTTTGTGCGTATATATTTGAGG-3′ and reverse: 5′-GGAAGATGTTGATGAGGT-3′.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ChIP, chromatin immunoprecipitation; Co-IP, coimmunoprecipitation; dnFIK, dominant-negative FOXP3-interacting KRAB domain–containing protein; FIK, FOXP3-interacting KRAB domain–containing protein; qPCR, quantitative PCR; siRNA, small interfering RNA; Treg, regulatory T cell.

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**Coimmunoprecipitation and Western blot**

Coimmunoprecipitation (Co-IP) and Western blot were performed as described previously (9).

**Chromatin immunoprecipitation assays**

Chromatin immunoprecipitation (ChIP) assays were performed according to the manufacturer’s instructions (Upstate Biotechnology, Lake Placid, NY). The following primers were used for ChIP: human IL-2 promoter, forward: 5'-CTCTTGCTCTTGTGCCACCC-3' and reverse: 5'-ATCCCTCTTGGTTACATTAGCC-3'; and human IFN-γ promoter, forward: 5'-CCAGTGTCCTTAATGGTGTGAAGT-3' and reverse: 5'-TAACCTAAGGGTTTGGTGCGCATTGGG-3'.

**Retroviral and lentiviral infection**

Full-length human FOXP3 and FIK were cloned into retroviral vectors, as described previously (11), or transfected with siRNA, as described above. Full-length human FIK or dominant-negative FIK (dnFIK) was cloned into lentivirus vector pCVL.MND.SceOPT.2A.GFP (11).

**RNA interference**

Small interfering RNA (siRNA) oligonucleotides specific to human FOXP3 (siRNA sequence: CACUCUAAUGAGAUCUAACAdTdT) and KAP1 (siRNA sequence: GCCUCUAUGGCGGCAACAdTdT) (Sigma-Aldrich) were introduced into primary human Treg by nucleofection using an Amaxa instrument (Lonza).

**Treg-suppressive assay**

CD4^+CD25^+CD45RA^+ primary human T cells were sort purified and transduced with lentiviral vectors, as described previously (11), or transfected with siRNA, as described above. Suppression activity was measured using CFSE-dilution assays, as described previously (12), or a BD FastImmune Regulatory siRNA, as described above. Suppression activity was shown by Co-IP from CD4^+CD25^+ human T cells (Fig. 1B).

**Results and Discussion**

To gain an understanding of the molecular basis of FOXP3-mediated transcriptional regulation, we performed a yeast 2-hybrid screen using the amino terminal 200 aa of FOXP3 as bait and human Treg cDNA as prey. This region was chosen because we showed previously that it contained sequences required for FOXP3 to act as a transcriptional repressor (13). Among the sequences that bound to this region of FOXP3 were RORα and RORγt, which provided important insights into the role of FOXP3 in the regulation of Treg/Th17 differentiation (9, 10). In addition, a clone that encoded a proline rich N-terminal of FOXP3, especially aa 106–198, is required to interact with FIK. Interestingly, this region of FOXP3 can also interact with Eos to mediate gene silencing in Tregs (20). In addition, Li et al. (21) showed that this region of FOXP3 is important for its ability to interact with Tip60 and histone deacetylases. Taken together, these data suggest that this region of FOXP3 is critical in regulating its association with repressive chromatin-remodeling complexes, possibly through interactions involving FIK. The unique C-terminal region of FIK is necessary and sufficient for interacting with FOXP3 (Supplemental Fig. 1C).

As mentioned previously, a major feature of FIK is the presence of a KRAB domain (Supplemental Fig. 2A). As described above, this domain, found primarily in about one third of eukaryotic Kruppel-type ZFPs, serves as a binding site for the scaffolding protein KAP1 (16, 17). Gene silencing requires the binding of the KRAB domain to the RING-B box-coiled coil domain of the coressor KAP1. KAP1 then represses gene transcription by recruiting the histone methyltransferase SETDB1, HP1, and the NuRD histone deacetylase complex (17). The KRAB domain in FIK contains both A box and B box; both are required for it to bind to KAP1 (Supplemental Fig. 2B). These data suggest that this region of FIK is critical in regulating its association with repressive chromatin-remodeling complex that contains KAP1. To test this hypothesis, we first asked whether FOXP3, FIK, and KAP1 formed a complex in cells. As shown in Fig. 2A, following cotransfection of expression plasmid encoding each protein,
immunoprecipitating KAP1 (anti-FLAG), FOXP3 could be co-immunoprecipitated. We also transduced the human T cell line Jurkat, which does not express FOXP3 or FIK but does express KAP1, with retroviruses encoding these genes to determine their role in regulating IL-2 and IFN-γ expression. After 3 d, cells transduced with both retroviruses were sorted and stimulated with PMA plus ionomycin for 4 h, and IL-2 and IFN-γ expression was analyzed. As shown in Fig. 2B, FOXP3 alone can suppress IL-2 and IFN-γ expression, whereas FIK alone cannot. However, cells transduced with both genes showed a significantly decreased level of IL-2 and IFN-γ expression, consistent with a role for FIK in FOXP3 function.

We next performed a ChIP assay to determine whether the FOXP3–FIK–KAP1 complex physically binds to a target gene promoter. Human CD4+CD25+ cells were isolated and ex-

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sessed in vitro. A ChIP assay was performed using Abs against FOXP3, FIK, and KAP1. As shown in Fig. 2C, we were able to immunoprecipitate all three proteins from the same site on the IL-2 promoter. Similar results were obtained using a site from the IFN-γ promoter (Fig. 2D). Taken together, these data demonstrate that FOXP3, FIK, and KAP1 can form a complex that is present on the promoters of FOXP3-repressed genes.

Next, we investigated whether blockade of FOXP3–FIK–KAP1 interaction in Tregs would affect gene expression and suppressor function in Tregs. We initially attempted to generate siRNAs to target FIK but were unsuccessful in finding sequences that led to demonstrable knockdown of FIK mRNA. We next took advantage of the fact that the C terminus of FIK was capable of binding to FOXP3 but that the entire KRAB domain was required for KAP1 interaction (Supplemental Figs. 1, 2). Using this information, we designed a form of FIK that would bind to FOXP3, but not to KAP1 (aa 75–113), and thus act as a dominant negative (dnFIK), competing with endoge-

FIGURE 2. FOXP3, FIK, and KAP1 form a transcription-suppressive complex in Tregs. (A) HEK 293T cells were transfected with plasmids, as indicated. Co-IP experiments were performed, as described, using anti-FLAG Ab. (B) Jurkat T cells were infected with retrovirus encoding FOXP3 and FIK, as described. After 3 d, double-positive cells were sorted and stimulated. IL-2 and IFN-γ transcription relative to GAPDH was quantified by qPCR analysis. Open bar: MIGR1+NGFR; filled bar: MIGR1+FIK; hatched bar: FOXP3+NGFR; bar represents FOXP3+FIK. Transcript level in the control group infected with MIGR1 plus NGFR was set as 1. (C and D) ChIP assays were performed as described in Materials and Methods. Goat anti-human FOXP3, goat anti-human KAP1, and rabbit anti-human FIK Abs were used to pull down the protein–DNA complexes, followed by qPCR analysis (C) or gel electrophoresis (D) using primers specific to human IL-2 (C) and IFN-γ (D) promoter. Data are mean ± SD and represent three independent experiments. **p < 0.01.

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FIGURE 3. FOXP3–FIK interaction is essential for Treg function. CD4+CD25+ Tregs were maintained in X-VIVO medium with anti-CD3/CD28 beads plus 300 U/ml IL-2. These cells were infected by lentivirus encoding full-length FIK or dnFIK or corresponding control virus. GFP+ cells were sorted after 4 d and analyzed. (A) Sorted GFP+ cells were stimulated with anti-CD3/CD28 beads for 4 h. Total RNA was reverse transcribed, and qPCR was performed to determine IL-2 and INF-γ expression. **p < 0.01. (B) Sorted GFP+ cells were mixed with CFSE-labeled PBMCs in the presence of anti-CD3 beads and cultured for 4 d. CFSE dilution of CD8+ cells was assessed by flow cytometry. UNTR represents untreated Tregs; pELNS-GFP represents Tregs infected by control virus, and pELNS-dnFIK represents Tregs infected by virus encoding dnFIK. The number beside the peak represents the suppressive activity of each group. (C) Summary of four independent suppressive assays. Data are mean ± SD and represent three independent experiments.

nous FIK for binding to FOXP3. A lentiviral vector encoding this fragment linked to GFP via a 2A sequence was used to transduce human CD4+CD25+ Tregs. Cells expressing dnFIK showed increased IL-2 and IFN-γ production following stimulation, two genes known to be repressed by FOXP3 (Fig. 3A). However, expression of genes that were shown to be positively

FIGURE 4. KAP1 is required for FOXP3 function. CD4+CD25+ Tregs were transfected with siRNA specific to human FOXP3 or KAP1 using Amaxa Human T Cells Transfection Kit. Cells were harvested after 3–4 d. (A) IL-2 and IFN-γ expression were determined by qPCR normalized to GAPDH expression. (B) IL-2 expression was also analyzed by intracellular staining. (C) ChIP assays were performed, as described. Rabbit anti-histone H3K9 (ab8898; Abcam) and rabbit anti-acetyl-histone H4 (17-630; Millipore) were used to pull down the protein–DNA complexes, followed by qPCR analysis using primers specific to human IL-2 promoter. (D) Suppressive assay was performed using a BD FastImmune Regulatory T-Cell Function Kit, according to the manufacturer’s instructions with minor modifications. Data are mean ± SD and represent three independent experiments.

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regulated by FOXP3 (CTLA-4 and CD25) was unaffected by dnFIK (data not shown).

We next determined whether inhibiting FOXP3–KAP1 interactions affected Treg function. For these studies, we again transduced activated human CD4+CD25+ T cells with the dnFIK lentivirus and sorted GFP+ cells. The infected Tregs were cultured at various ratios with CFSE-labeled PBMCs and stimulated with anti-CD3 beads. Proliferation was measured by CFSE dilution of CD8+ T cells. We found that Tregs expressing dnFIK had a dramatically reduced ability to suppress the proliferation of conventional CD8 T cells (Fig. 3B, 3C), showing that FOXP3–FIK–KAP1 interactions play an important role in human Treg function.

These studies were extended to directly determine the role of KAP1 in human Tregs, targeting FOXP3 and KAP1 with siRNAs. Human CD4+CD25+ T cells were expanded in vitro with open chromatin (acetylated histone H4) and decreased promoter displayed an increase in epigenetic marks consistent (2A, 4B). Concomitant with this increased expression, the IL-2 promoter displayed an increase in epigenetic marks consistent with open chromatin (acetylated histone H4) and decreased trimethyl histone H3K9, indicative of closed chromatin (Fig. 4C). Finally, we determined the ability of the FOXP3− and KAP1−/− Tregs resulted in increased expression of IL-2 and IFN-γ (Fig. 4A, 4B). Concomitant with this increased expression, the IL-2 promoter displayed an increase in epigenetic marks consistent with open chromatin (acetylated histone H4) and decreased trimethyl histone H3K9, indicative of closed chromatin (Fig. 4C). Finally, we determined the ability of the FOXP3− and KAP1−/− Tregs to suppress T cell proliferation in vitro. Both cell populations had a dramatic reduction in suppressor function in vitro (Fig. 4D). These data demonstrate that KAP1 is important for FOXP3 and Treg function.

KAP1 is a ubiquitously expressed multifunctional protein that was shown to be a critical factor for chromatin stability in both humans and mice (17). Recently, Chikuma et al. (23) reported that mouse T cells require Kap1 for proper cell-cycle progression in response to TCR stimulation. In addition, they showed that mice with T cell–specific deletion of Kap1 develop spontaneous autoimmunity (23). However, Tregs from these mice displayed an increase in epigenetic marks consistent with open chromatin (acetylated histone H4) and decreased trimethyl histone H3K9, indicative of closed chromatin (Fig. 4C). Finally, we determined the ability of the FOXP3− and KAP1−/− Tregs to suppress T cell proliferation in vitro. Both cell populations had a dramatic reduction in suppressor function in vitro (Fig. 4D). These data demonstrate that KAP1 is important for FOXP3 and Treg function.

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Disclosures

The authors have no financial conflicts of interest.

References

Figure S1: Cloning of human *FIK* gene and Mapping the interacting regions between FOXP3 and FIK.  

**A.** Nucleotide and deduced amino acid sequences of *FIK* gene (GenBank accession number KC249978). The nucleotide sequence of the 714 bp cDNA was shown in the top lines, and its predicted amino acid sequence was shown below in single-letter code. The ORF extended from nucleotide 190 to 531 and encodes a protein of 113 amino acids. The start codon is marked in italic type, and an asterisk indicates the terminator in the protein sequence. The KRAB domain is shaded. Sequences unique to FIK are underlined.  

**B.** Mapping the FIK interacting domain in FOXP3. HEK 293T cells were transfected with truncated forms of human FOXP3 and EGFP-FIK. After 36 hours, cell lysates were immunoprecipitated with anti-GFP antibody. Western blot were performed to detect both FOXP3 (by anti-DBD antibody) and FIK (by anti-GFP antibody).  

**C.** Mapping the FOXP3 interacting domain in FIK. HEK 293T cells were transfected with truncated forms of human FIK and FLAG-FOXP3. After 36 hours, cell lysates were immunoprecipitated with anti-FLAG antibody. Western blot were performed to detect both FOXP3 (by anti-FLAG antibody) and FIK (by anti-DBD antibody).  

Data represent three or more independent experiments.

Figure S2: Construction of human FIK plasmids and mapping the KAP1 interacting regions within FIK.  

**A.** Construction of full length and truncated forms of human FIK.  

**B.** HEK 293T cells were transfected with plasmids as indicated. Co-IP experiments were performed as described using anti-DBD antibody.  

Data
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