Ikaros Enforces the Costimulatory Requirement for IL2 Gene Expression and Is Required for Anergy Induction in CD4+ T Lymphocytes

Rajan M. Thomas, Neelanjana Chunder, Chunxia Chen, Sarah E. Umetsu, Susan Winandy and Andrew D. Wells

J Immunol 2007; 179:7305-7315; doi: 10.4049/jimmunol.179.11.7305
http://www.jimmunol.org/content/179/11/7305
T cell activation results in dynamic remodeling of the chromatin at the IL2 promoter and induction of IL2 gene transcription. These processes are each dependent upon CD28 costimulation, but the molecular basis for this requirement is not clear. The IL2 promoter contains consensus-binding elements for Ikaros, a lymphocyte-specific zinc-finger DNA-binding protein that can regulate gene expression by recruiting chromatin-remodeling complexes. We find that native Ikaros in CD4+ T cells exhibits sequence-specific binding to these elements in vitro, and interacts with the endogenous IL2 promoter in vivo, in a manner dependent upon its DNA-binding domain. This binding has important consequences on the regulation of the IL2 gene, because CD4+ T cells with reduced Ikaros DNA-binding activity no longer require signals from the TCR or CD28 for histone acetylation at the endogenous IL2 promoter, and no longer require CD28 costimulation for expression of the IL2 gene. Furthermore, CD4+ T cells with reduced Ikaros activity are resistant to clonal anergy induced by TCR ligation in the absence of either CD28 or IL-2R signals. These results establish Ikaros as a transcriptional repressor of the IL2 gene that functions through modulation of chromatin structure and has an obligate role in the induction of anergy.
and DNA methylation (NURD) complex (34, 35), as a major regulator of chromatin structure and transcription at the Ikaros locus. We demonstrate that Ikaros sets the CD28 costimulatory requirement in CD4+ T cells and is necessary for the induction of anergy in response to CD28 or IL-2R blockade. Therefore, Ikaros is crucial not only for the T lymphocyte lineage decision in the thymus (36), but also for the adoption of tolerant cell fates in mature CD4+ T cells.

**Materials and Methods**

**Reagents, Abs, and cells**

mAbs against murine CD3 (145-2C11) and CD28 (37.51) and CTLA-4g fusion protein were generated and purified by BioExpress. mAbs against CD25, CD122, and CD132 were purchased from BD Biosciences. mAbs against Ikaros were a gift of K. Georgopoulos (Harvard Medical School, Cambridge, MA), and affinity-purified polyclonal antisera against Ikaros were purchased from Santa Cruz Biotechnology. Anti-FLAG epitope Ab and polybrene were purchased from Sigma-Aldrich. mAbs against HDAC1 and HDAC2, as well as affinity-purified polyclonal antisera against acetylated histone H3, were purchased from Upstate Biotechnology. PCR primers were synthesized by GeneLink. Routine molecular biology reagents were purchased from Sigma-Aldrich. The Ikarosmi7 thymoma cell line, JEl31, and murine stem cell virus-based retroviral vectors encoding FLAG-tagged Ikaros-1 (MIGR1-FLAG-Ik1) and Ikaros-7 (MIGR1-FLAG-Ik7), were characterized previously (37).

**Mice**

C57BL/6 mice were purchased from The Jackson Laboratory. The transgenic Ik7 dominant-negative transgenic mice (Ik7DN) on a C57BL/6 background (38) were obtained from K. Georgopoulos (Harvard Medical School, Boston, MA). Male mice with one copy of the transgenic Ik7 allele were bred with wild-type B6 females to generate offspring with only one copy of the dominant-negative allele (Ik7DN/+), which were identified by PCR genotyping. Ik7DN/+ and Ikarosmi7 mice in these studies were used at 5–7 wk of age, before the development of CD4+CD8+ thymocytes. Absence of thymocyte cells, which do not express a functional TCR (37) and do not produce IL-2, was confirmed in each experiment by CD4/CD8 staining before use. IL2GFP-transgenic mice (4) were obtained from E. Rothenberg (Caltech, Pasadena, CA) and bred against IkDN/+ mice. All mouse procedures were conducted according to protocols and guidelines approved by the Joseph Stokes, Jr. Research Institutional Animal Care and Use Committee.

**Lymphocyte culture and purification**

Single-cell suspensions of lymph node and spleen cells were prepared, depleted of CD8+ cells using CD8 microbeads (Miltenyi Biotec), and stimulated in 24-well plates at 2 × 10^6 cells/ml with soluble anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml) or CTLA-4g (5 μg/ml) in RPMI 1640 supplemented with 10% FBS, penicillin/streptomycin, t-glutamine, and 50 μM 2-ME. Cells were harvested at the time points indicated in each figure, washed in PBS, and CD4+ T cells were purified by positive selection using anti-CD4-coated microbeads (Miltenyi Biotec). The isolated CD4+ T cells were 92–96% pure in these experiments. In restimulation experiments, primed cells were allowed to rest for 24 h in medium, then stimulated in 24-well plates with immobilized anti-CD3 (1 μg/ml).

**ChIP primers:**

<table>
<thead>
<tr>
<th>IL2 A region (~698 to ~466)</th>
<th>Forward 5'-AGAAGCAACCCCTTCTGAAGA-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL2 B region (~467 to ~227)</td>
<td>Reverse 5'-ACCTTTCTTACTAACAATGACG-3'</td>
</tr>
<tr>
<td>IL2 promoter (~232 to ~99)</td>
<td>Forward 5'-TGATACACCATCTGGAAGAACC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CAGAGACCAAGTGCTAACTTGGA-3'</td>
</tr>
<tr>
<td>CD3e promoter</td>
<td>Forward 5'-CTGCTCTCAACATTCTCAAGTGA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-ACACACTGTGGCTGAAGAAAGG-3'</td>
</tr>
<tr>
<td>γ-satellite repeat</td>
<td>Forward 5'-TGGGCAAGAANATCGAAAAGG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CTGGCTATTCCAGGTTCCTT-3'</td>
</tr>
<tr>
<td>Brgl intron</td>
<td>Forward 5'-CCTGTTGTCCTAACACTGATAAG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GAGTCCTCCCCCTAGGACCAGTAAG-3'</td>
</tr>
</tbody>
</table>

**EMSA probes:**

- 203 site: Sense 5'-AACCCGGACCAAGGAGGATTTCACCTAAATC-3'
- 17 site: Sense 5'-AAATGGCTTCCCTACTGGAAGAGCTGCTCTA-3'
- Mutated -203 site: Sense 5'-AACCCGACCAAGGAGGATTTCACCTAAATC-3'

*Mutated nucleotides are underlined.

**Table I. Primers and oligonucleotide probes used for experiments**

<table>
<thead>
<tr>
<th>Primers and Probes</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChIP primers:</td>
<td></td>
</tr>
<tr>
<td>IL2 A region (~698 to ~466)</td>
<td>Forward 5'-AGAAGCAACCCCTTCTGAAGA-3'</td>
</tr>
<tr>
<td>IL2 B region (~467 to ~227)</td>
<td>Reverse 5'-ACCTTTCTTACTAACAATGACG-3'</td>
</tr>
<tr>
<td>IL2 promoter (~232 to ~99)</td>
<td>Forward 5'-TGATACACCATCTGGAAGAACC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CAGAGACCAAGTGCTAACTTGGA-3'</td>
</tr>
<tr>
<td>CD3e promoter</td>
<td>Forward 5'-CTGCTCTCAACATTCTCAAGTGA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-ACACACTGTGGCTGAAGAAAGG-3'</td>
</tr>
<tr>
<td>γ-satellite repeat</td>
<td>Forward 5'-TGGGCAAGAANATCGAAAAGG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CTGGCTATTCCAGGTTCCTT-3'</td>
</tr>
<tr>
<td>Brgl intron</td>
<td>Forward 5'-CCTGTTGTCCTAACACTGATAAG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GAGTCCTCCCCCTAGGACCAGTAAG-3'</td>
</tr>
</tbody>
</table>

Chromatin immunoprecipitation (ChIP) analysis

In vivo binding of Ikaros to the γ-satellite, Brgl-1, CD8α, IL2, and CD3e chromosomal regions was examined by ChIP analysis using anti-FLAG Ab or a mixture of anti-Ikaros Abs. The γ-satellite, Brgl-1, and CD8α genes have been defined to bind Ikaros in vivo (Refs. 40 and 41 and T. Naito and K. Georgopoulos, unpublished observations), and serve as positive controls for our Ikaros ChIP analyses. Analysis of histone acetylation at these regions was performed using Ab specific for lysine-acetylated histone H3 (Upstate Biotechnology). Chromatin-nuclear protein complexes from purified CD4+ T cells were fixed with 1% formaldehyde, sheared into 150- to 700-bp fragments by sonication, and the ChIP procedure was performed as described previously using a ChIP assay kit from Upstate Biotechnology (5, 42). Control ChIP reactions using irrelevant Abs were performed for each experiment, and all ChIP reactions were performed in duplicate or triplicate. Genomic DNA was detected by quantitative real-time PCR, in triplicate, using primer sets listed in Table I. Relative Ikaros binding and histone acetylation were calculated as a specific signal-to-noise ratio, as described previously (5).
Retroviral transduction

Murine stem cell virus-based bicistronic retroviral encoding GFP and Ikaros (MIGR1-Ik) were generated by transient cotransfection of the Phoenix packaging cell line (provided by G. Nolan, Stanford University, Stanford, CA) with MIGR1-Ik plasmid and pCLeco helper plasmid (Imgenex). Cells were transfected by incubating the cells in antibiotic- and serum-free medium with DNA-Lipofectamine 2000 (Invitrogen Life Technologies) complexes for 6 h at 37°C, followed by 48 h culture in complete medium. Purified CD4+ T cells were activated for 16 h with PMA (3 ng/ml), ionomycin (1 μM), and IL-2 (10 U/ml). For transduction, activated T cells were resuspended in 48-h viral supernatant from Phoenix cultures (0.5 μg/ml), and centrifuged at 2500 rpm for 90 min. Transduced cells were expanded in medium with DNA-Lipofectamine 2000 (Invitrogen Life Technologies) complexes for 6 h at 37°C, followed by 48 h culture in complete medium. Purified CD4+ T cells were activated for 16 h with PMA (3 ng/ml), ionomycin (1 μM), and IL-2 (10 U/ml). For transduction, activated T cells were resuspended in 48-h viral supernatant from Phoenix cultures (0.5 × 10^6 cells/ml viral supernatant in 24-well plates) with polybrene (8 μg/ml), and centrifuged at 2500 rpm for 90 min. Transduced cells were expanded in IL-2 (5 U/ml) for 72 h and rested before use in experiments.

Quantitative real-time PCR

Quantitative real-time PCR was performed in triplicate for each mRNA or ChIP sample using primers indicated in Table I. SYBR-Green PCR master mix was purchased from Applied Biosystems.

Immunoblot analysis

Nuclear extracts were prepared from CD4+ T cells using an extraction kit (Pierce), and whole cell extracts of transduced HEK131 cells were prepared in SDS-loading buffer. Five micrograms of extract was subjected to SDS-PAGE and transferred to nitrocellulose membrane by electroblotting. Blots were probed with anti-Ikaros C-terminal Ab (Santa Cruz Biotechnology), anti-FLAG (Sigma-Aldrich), or anti-actin (Sigma-Aldrich).

Flow cytometry and ELISA

GFP expression by CD4+ T cells was assessed by flow cytometry using a Cyan cytometer (DakoCytomation). IL-2 secreted by the activated T cells was detected by a mouse IL-2 ELISA kit from eBioscience and following guidelines provided by the vendor.

Results

Ikaros binds in a sequence-specific manner to the IL2 promoter in vitro

A scan of the DNA sequence upstream of the IL2 gene revealed the presence of two putative Ikaros consensus DNA-binding elements within the −300-bp minimal promoter/enhancer region (Fig. 1A). One of these consensus elements is located on the antisense strand 17 bp upstream of the transcription start site, in close proximity to the primary TATA box (Fig. 1A, −17 site). The other element is located at −203 bp, overlapping a defined NF-κB-binding site within the distal T cell element (TCε)IL-2B footprint nuclear-binding factor (NFIL)-2C region (Fig. 1A, −203 site).

To determine whether Ikaros can bind to sequences derived from the IL2 promoter/enhancer in vitro, we performed EMSA analysis using double-stranded oligonucleotides comprising the Ikaros consensus elements at −203 and −17 bp (Table I) to probe nuclear extracts of resting CD4+ T cells from naive mice. These probes each shifted two specific complexes (Fig. 1B, lanes 1, 2, 3, 5, and 6). The −203 probe consistently exhibited a higher binding affinity than the −17 probe in our EMSA analyses. To confirm that the Ikaros core-binding motif (GGGA) was required for the formation of these complexes, we introduced a point mutation in the −203 probe that substituted the middle guanine with a cytosine (GGGA). This point mutation underlined resulted in a nearly complete loss of the bound complex (Fig. 1B, lanes 7 and 8).

To confirm that these DNA-nuclear protein complexes contain Ikaros, we took two approaches. First, we used anti-Ikaros Abs to disrupt the native oligomeric form of Ikaros that is responsible for DNA binding in the context of our EMSA analysis (43). Abs specific for either the C terminus or the N terminus of Ikaros caused a marked reduction in the bound complexes formed with both the −203 and the −17 probes (Fig. 1B, lanes 9–13). Second, we used the −203 core element to probe nuclear extracts of CD4+ T cells expressing the Ik7 isoform of Ikaros that lacks the N-terminal zinc finger domains (38), which are required for DNA binding (43, 44). These nuclear extracts exhibited a marked decrease in the bound complexes compared with extracts of wild-type cells (Fig. 1B, lanes 14 and 15), confirming that the high-affinity complexes observed in these experiments is dependent upon Ikaros DNA-binding activity. Together, these data demonstrate that Ikaros present in nuclear extracts from naive CD4+ T cells can bind in vitro to the consensus Ikaros core elements present in the IL2 promoter/enhancer at −203 and −17 bp.
Ikaros binds to the endogenous IL2 promoter/enhancer in vivo

To determine whether Ikaros binds to the endogenous IL2 locus in CD4+ T cells in vivo, we used ChIP analysis to measure in situ occupancy of Ikaros at various regions of the genome, including the IL2 promoter/enhancer region. We prepared chromatin-DNA complexes from resting CD4+ T cells from naive mice and immunoprecipitated these extracts with a mixture of anti-Ikaros Abs. As a positive control, we probed the precipitated genomic DNA from these ChIP samples for centromeric γ-satellite repeat sequences, as a large proportion of Ikaros is colocalized with centromeric heterochromatin in lymphoid cells (40). These sequences were highly enriched in the Ikaros ChIP samples compared with a 10% aliquot of unprecipitated genomic DNA, confirming the efficacy of our anti-Ikaros ChIP procedure (Fig. 2A). These ChIP samples were likewise probed for several regions upstream of the IL2 gene. This analysis showed significant enrichment of genomic DNA-chromatin complexes containing the defined Ikaros-binding elements in the promoter/enhancer of the IL2 gene (Fig. 2B, gray triangles in region P), and significantly less enrichment of regions further upstream that do not contain putative Ikaros-binding sites (Fig. 2B, regions A and B). We extended these results in additional ChIP experiments using a quantitative real-time PCR approach. These anti-Ikaros ChIP analyses demonstrated >4-fold enrichment of the defined Ikaros-binding site in the intronic region of the Brg1 locus (Fig. 2C, light gray bars), and >5-fold enrichment of the IL2 promoter/enhancer region (Fig. 2C, dark gray bars), compared with the control nonspecific Ab ChIP (Fig. 2C, IgG).

To further confirm the above ChIP results and to determine whether the DNA-binding domain of Ikaros is required for in vivo IL2 promoter occupancy, we performed Ikaros ChIP analyses on Ikarosnull JE131 cells reconstituted by retroviral transduction with either full-length tagged Ikaros (Fig. 3A, FLAG-Ik1), or with the Ik7 isoform lacking the N-terminal zinc finger domains required for DNA binding (Fig. 3A, FLAG-Ik7). Transduction was confirmed by GFP fluorescence (Fig. 3B), and expression of each Ikaros construct was confirmed by immunoblotting (Fig. 3C). Abs against either Ikaros or the FLAG tag enriched the positive control Brg1 and CD8α genes 15- to 20-fold from FLAG-Ik1-expressing JE131 cells as compared with extracts from JE131 cells transduced with empty vector (Fig. 3, D and E). Likewise, the ChIP samples from FLAG-Ik1-transduced JE131 cells showed a 10- to 20-fold enrichment of the IL2 promoter/enhancer region over background.

FIGURE 2. ChIP analysis of in vivo IL2 promoter occupancy by Ikaros. A, Binding of Ikaros to centromeric γ-satellite repeat regions in resting CD4+ T cells from naive mice. Left lane, Control amplification of γ-satellite DNA from an aliquot of unprecipitated chromatin (input); right lane, γ-satellite DNA amplified from chromatin enriched for Ikaros-bound complexes using anti-Ikaros Abs (Ikaros ChIP). Each reaction was run for 32 cycles. B, Schematic representation of the IL2 locus depicting Ikaros consensus elements (gray triangles) and the three primer sets used to probe for the promoter/enhancer (P) and two regions further upstream of the transcriptional start site (A and B). The first three lanes show DNA amplified from chromatin precipitated with anti-Ikaros, and the last three lanes depict amplification from input chromatin prepared from naive CD4+ T cells. C, Quantitative real-time PCR quantification of Ikaros binding to the endogenous Brg1 intron (light gray bars) and IL2 promoter/enhancer (dark gray bars). Specific Ikaros binding is calculated as the ratio of the specific anti-Ikaros ChIP signal to the background isotype control Ab ChIP signal.

FIGURE 3. Influence of DNA-binding activity on IL2 promoter occupancy by Ikaros. A. Schematic representation of MIGRI-based retroviral vectors encoding FLAG-tagged Ikaros-1 and Ikaros-7 splice variants. B, Reporter gene (GFP) expression by Ikarosnull JE131 thymoma cells (light gray histogram) transduced with MIGRI-Ik1 (dark gray histogram) and MIGRI-Ik7 (black histogram). C, FLAG immunoblot analysis of Ik1 and Ik7 expression in transduced JE131 cells (top panel). Blots were also probed for actin (bottom panel). D–F, Chromatin prepared from Ikarosnull JE131 thymoma cells transduced with empty vector, FLAG-Ik1, or FLAG-Ik7 was precipitated with Abs against either the FLAG epitope (light gray bars) or Ikaros (dark gray bars). ChIP samples were probed for the Brg1 intron (D), CD8α promoter (E), or the IL2 promoter/enhancer (F). Results are representative of two to three separate experiments.
CD4+ T cells (left panels) from naive wild-type B6 (top panels) or B6-Ik7DN/+ (bottom panels) mice were stimulated with anti-CD3 Ab plus CTLA-4Ig (middle panels) or anti-CD28 (right panels). Chromatin prepared from these cells was immunoprecipitated with isotype control or anti-AcH3 Ab, and precipitated DNA was probed for regions upstream of the IL2 gene (A and B) or for the CD3ε promoter (C). AcH3 in B and C was calculated as the ratio of the specific anti-AcH3 ChIP signal to the background isotype control Ab ChIP signal. Results are representative of four separate experiments. D, Nuclear extracts of resting CD4+ T cells from naive mice were probed with the −203 consensus oligo in the presence (lane 3) or absence (lane 2) of anti-HDAC1 and anti-HDAC2 Abs. Data depicted are representative of two independent experiments.

Our previous studies have shown that histone acetylation and chromatin remodeling upstream of the IL2 gene in CD4+ T cells is dependent upon signals from both the TCR and CD28 (5). Consistent with these results, we found that the nucleosomes positioned across the IL2 promoter/enhancer in wild-type naive CD4+ T cells were hypoacetylated (Fig. 4, top left panel in A, first dark gray bar in B). Similarly, the histones at the IL2 promoter/enhancer remained hypoacetylated following TCR stimulation in the absence of CD28 costimulation (Fig. 4, top middle panel in A, second dark gray bar in B), and only the combination of TCR and costimulation was able to induce histone acetylation at this locus (Fig. 4, top right panel in A, third dark gray bar in B). However, CD4+ T cells with reduced Ikaros DNA-binding activity did not exhibit these same requirements for histone acetylation at the IL2 locus. Like wild-type cells, CD4+ T cells from Ik7DN/+ mice exhibited strong acetylation of histone H3 at the IL2 promoter/enhancer in response to TCR/CD28 costimulation (Fig. 4, bottom right panel in A, third light gray bar in B). Unlike wild-type cells, however, Ik7DN/+ CD4+ T cells did not require CD28 signals to achieve histone hyperacetylation (Fig. 4, bottom middle panel in A, second light gray bar in B), nor did they require signals from the TCR, as resting CD4+ T cells from naive Ik7DN/+ mice also exhibited the same degree of AcH3 at the IL2 promoter/enhancer as TCR/CD28 costimulated T cells (Fig. 4, bottom left panel in A, first light gray bar in B). This dysregulated pattern of histone acetylation was specific for the IL2 locus, as the CD3ε promoter was not hyperacetylated in Ik7DN/+ CD4+ T cells as compared with wild-type cells (Fig. 4C). These data suggest that Ikaros normally acts to recruit histone deacetylase activity to the IL2 promoter/enhancer. Consistent with this, the inclusion of Abs against HDAC1 and HDAC2 disrupted the formation of Ikaros-containing complexes from wild-type CD4+ T cell nuclear extracts with the −203 Ik-binding site probe, as analyzed by EMSA (Fig. 4D). Together, these results show that Ikaros binds to the IL2 promoter/enhancer as part of a complex containing HDAC1 and/or HDAC2, and that in situ Ikaros occupancy is required to maintain basal histone hypoacetylation at the IL2 locus in quiescent and anergic CD4+ T cells.

Ikaros represses IL2 gene expression in mature CD4+ T cells

Our results so far have shown that Ikaros binds to the IL2 promoter/enhancer in situ and maintains the nucleosomes in this region in a hypoacetylated state until the appropriate signals (i.e., TCR and CD28) are received. We were therefore interested in whether this activity of Ikaros has functional consequences for the normal, context-dependent pattern of IL2 gene expression in CD4+ T cells. To do this, we measured IL-2 mRNA and protein secretion by CD4+ T cells from wild-type vs Ik7DN/+ mice following activation with soluble, agonistic Abs against the TCR complex and CD28. Under these conditions, CD4+ T cells with reduced Ikaros DNA-binding activity exhibited a 2- to 4-fold increase in the abundance of IL-2 mRNA and protein compared with wild-type CD4+ T cells (Fig. 5, A and B). Similarly, purified CD4+ T cells from Ik7DN/+ mice produced 4-fold more IL-2 protein than wild-type cells when stimulated with plate-bound Abs against the TCR and CD28 (Fig. 5C).
To determine whether Ikaros influences the frequency of activated T cells that are capable of producing IL-2, we bred the Ik7DN allele into mice transgenic for GFP driven by the upstream regulatory elements of the IL2 locus (4). CD4+ T cells carrying the Ik7DN allele exhibited 3- to 4-fold higher frequencies of GFP+ cells throughout the entire response to CD3/28 Ab stimulation as compared with IL2GFP-transgenic mice with wild-type Ikaros activity (Fig. 5D). These data demonstrate that Ikaros functions in normal CD4+ T cells to limit IL2 gene expression, even under optimal stimulatory conditions.

Our results in Fig. 4 demonstrate that CD4+ T cells with reduced Ikaros DNA-binding activity no longer require signals from the TCR or CD28 for histone hyperacetylation at the IL2 promoter/enhancer. Because histone acetylation is thought to promote gene transcription, we tested whether CD4+ T cells with reduced Ikaros DNA-binding activity likewise exhibit altered requirements for TCR and/or costimulatory signals for expression of the IL2 gene. Like wild-type cells, quiescent CD4+ T cells from naive Ik7DN+/mice did not express IL-2 mRNA or protein (data not shown), therefore the lack of Ikaros DNA-binding activity is not sufficient to allow IL2 transcription in the absence of antigenic signals. As expected, wild-type CD4+ T cells showed little or no expression of the IL2 gene when stimulated in the absence of CD28 costimulation (Fig. 5, E and F, dark gray symbols), however, stimulation of Ik7DN+/ CD4+ T cells under the same conditions resulted in a 10- to 20-fold greater expression of the IL2 gene (Fig. 5, E and F, light gray symbols), and a 2- to 3-fold higher frequency of IL-2 producers than in wild-type cultures (Fig. 5H). Similarly, while purified wild-type CD4+ T cells stimulated through the TCR alone produced little detectable IL-2 (Fig. 5G, dark gray bar), purified Ik7DN+/ CD4+ T cells produced large amounts of IL-2 under these conditions (Fig. 5G, light gray bar). These results confirm that the influence of Ikaros on IL-2 production is fully intrinsic to CD4+ T cells. Importantly, in all these experiments, induction of both IL-2 mRNA and protein by Ik7DN+/ CD4+ T cells in the absence of CD28 costimulation was equal to or greater than the levels induced in wild-type T cells by stimulation through the TCR and CD28 together (Fig. 5G, and insets in E and F). These data show that expression of the IL2 gene in CD4+ T cells is rendered CD28 independent in the absence of Ikaros DNA-binding activity.

To test whether the functional effects of dominant-negative Ikaros reflect a bona fide loss of Ikaros function, we assessed IL2 gene expression by the few CD4+ T cells that develop in mice that entirely lack the Ikaros gene (Ikarosnull). Indeed, like T cells from Ik7DN+/mice, Ikarosnull CD4+ T cells exhibited strong overproduction of IL-2 in response to TCR ligation (Fig. 6A), implying that the effects of dominant-negative Ikaros expression on IL2 gene expression are not due to cross-inhibition of other related zinc finger proteins.

Because the CD4+ T cells from Ikarosnull and Ik7DN+/ mice develop in the absence of normal Ikaros activity, we were concerned that the enhanced IL2 gene expression exhibited by these cells might be due to altered “tuning” in the thymus, and not due to an autonomous defect in Ikaros activity during peripheral activation. To address this issue, we transduced wild-type, peripheral CD4+ T cells with retroviral vectors encoding either full-length Ik1, or the Ik7 variant lacking the DNA-binding domain (Fig. 6B). Wild-type CD4+ T cells transduced with Ik1 exhibited a 3-fold
decrease in \( IL2 \) gene expression compared with mock- or empty vector-transduced cells (Fig. 6C), which was likewise accompanied by a \( \sim 3 \)-fold decrease in histone acetylation at the \( IL2 \) promoter (Fig. 6D). Conversely, CD4\(^+\) T cells transduced with Ik7 showed a >2-fold increase in IL-2 in response to AgR stimulation (Fig. 6C), which was likewise accompanied by augmented histone acetylation at the \( IL2 \) promoter (Fig. 6D). These data show that Ikaros expressed in mature CD4\(^+\) T cells acts directly to block histone acetylation and inhibit the expression of the \( IL2 \) gene. Together, these results demonstrate that Ikaros is a transcriptional repressor of the \( IL2 \) locus that participates in setting the costimulatory requirement for \( IL2 \) gene expression in CD4\(^+\) T cells.

**FIGURE 6.** Modulation of Ikaros activity in mature CD4\(^+\) T cells. A. Purified CD4\(^+\) T cells from wild-type or Ikaros\(^{-/-}\) mice were stimulated with varying amounts of anti-CD3 Ab coinimmobilized with 4 \( \mu \)g/ml anti-CD28 Ab, and IL-2 was measured by ELISA 6 h later. B. Wild-type CD4\(^+\) T cells were transduced with empty MIGR1 retroviral vector, or MIGR1 encoding Ik1 or Ik7, and CD4\(^+\)GFP\(^+\) cells were purified by FACS. C. After 24 h of rest, the transduced cells were restimulated for 6 h with plate-bound anti-CD3 (1 \( \mu \)g/ml), and IL-2 production was measured by ELISA. The data depicted are representative of four separate experiments. D. In separate experiments, chromatin prepared from transduced CD4\(^+\) T cells was subjected to ChIP analysis for histone acetylation (AcH3) at the \( IL2 \) promoter. The data depicted are representative of two separate experiments.

**FIGURE 7.** Ikaros DNA-binding activity promotes anergy in CD4\(^+\) T cells. A. CD8-depleted spleen cells from naïve wild-type (dark gray symbols in C) or Ik7DN\(^{-/-}\) (light gray symbols in C) mice were stimulated with anti-CD3/CD28 to induce effector differentiation (squares in C), or anti-CD3/CTLA-4Ig to induce anergy (circles in C). B. Nuclear extracts from purified CD4\(^+\) T cells were prepared and subjected to immunoblot analysis using a C-terminal-specific anti-Ikaros Ab to assess Ikaros splice variant expression. C. CD4\(^+\) cells were purified, rested for 24 h, restimulated on plate-bound anti-CD3, and IL-2 production was measured at the indicated time points by ELISA. Comparable results were observed when restimulation was performed with anti-CD3 plus anti-CD28 Ab (data not shown). D. Resting CD4\(^+\) T cells from naïve wild-type (dark gray symbols in E and F) or Ik7DN\(^{-/-}\) (light gray symbols in E and F) mice were stimulated with anti-CD3 in the presence or absence of CTLA-4Ig and/or neutralizing Ab against IL-2 (E), or neutralizing Ab against IL-2, IL-2Ra, IL-2R\(\beta\), and IL-2R\(\gamma\) (F). CD4\(^+\) cells were purified, rested for 24 h, restimulated on plate-bound anti-CD3, and IL-2 production was measured at 6 h (E) or 20 h (F) by ELISA. All results above are representative of two separate experiments.
Ikaros DNA-binding activity is required for T cell anergy

CD28 costimulation and IL-2 production are each crucial factors in the avoidance of T cell clonal anergy (13). Given the role defined above for Ikaros in setting the CD28 costimulatory threshold for IL-2 production in activated CD4+ T cells, we hypothesized that Ikaros may be required for the induction of anergy. To test this, we stimulated wild-type or Ik7DN/+ CD4+ T cells in the presence vs absence of CD28 costimulation, allowed the cells to rest, and assessed IL-2 production by these cells in response to restimulation through the TCR (Fig. 7A).

First, because the Ikaros gene can be expressed as eight distinct splice variants with differential DNA-binding activity, we characterized the pattern of Ikaros protein expression in unstimulated, effector, and anergic CD4+ T cells. Quiescent CD4+ T cells and cells stimulated in the presence vs absence of costimulation all showed roughly comparable nuclear expression of the DNA-binding Ik1 and Ik2 isoforms, and little or no expression of N- or C-terminal-truncated isoforms (Fig. 7B, lanes 1–3). This confirms that Ikaros is not an anergy-specific factor in our system, but is expressed and acts to repress IL2 gene expression in T cells at multiple stages of differentiation (see Figs. 5 and 6). As expected, CD4+ T cells from IkDN/+ transgenic mice predominantly expressed the Ik7 form in the nucleus, independent of stimulatory conditions (Fig. 7B, lanes 4–6).

Wild-type CD4+ T cells primed through the TCR and CD28 produced high levels of IL-2 upon restimulation (Fig. 7C, dark gray squares), while wild-type CD4+ T cells stimulated in the absence of CD28 costimulation displayed an anergic phenotype, with markedly reduced IL-2 production upon restimulation (Fig. 7C, dark gray circles). Ik7DN/+ CD4+ T cells primed through the TCR and CD28 showed a slight increase in IL-2 production upon restimulation (Fig. 7C, light gray squares), but unlike wild-type cells, CD4+ T cells with reduced Ikaros DNA-binding activity were strongly resistant to anergy induction, exhibiting ~1000-fold greater IL-2 production than anergic wild-type CD4+ T cells and approaching the levels expressed by wild-type effector cells (Fig. 7C, light gray circles).

IL-2 is a potent T cell growth factor that can replace CD28 signals for proliferation and anergy avoidance during T cell activation (47–50). Because Ik7DN/+ CD4+ T cells produce IL-2 in a CD28-independent manner during primary stimulation (Fig. 5), it is possible that the resistance of Ikaros-mutant T cells to anergy induction is a direct consequence of this “inappropriate” IL-2 bioactivity. To test this, we neutralized IL-2 during primary stimulation of wild-type and Ik7DN/+ CD4+ T cells, and restimulated without modulation of IL-2 signaling (Fig. 7D). Inhibition of IL-2/IL-2R-mediated signal transduction was as potent as CD28 blockade for the induction of anergy in wild-type CD4+ T cells, while combined blockade of CD28 and IL-2 essentially eliminated secondary IL-2 production by wild-type cells (Fig. 7E, dark gray bars). Remarkably, Ikaros-mutant CD4+ T cells primed in the absence of either CD28- or IL-2-mediated signals produced as much IL-2 upon restimulation as mutant cells primed in the presence of these signals (Fig. 7E, light gray bars). Growth factor signaling through the common γ-chain has been shown to mediate anergy avoidance in human T cell clones (51). To test whether growth factor receptor-coupled signaling pathways are necessary for anergy resistance in Ikaros-mutant T cells, we stimulated wild-type vs Ik7DN/+ CD4+ T cells in the presence of Abs that block growth factor binding to the α-, β-, and γ-chains of the IL-2R. As with neutralization of IL-2, blockade of growth factor receptor binding led to profound anergy in wild-type CD4+ T cells (Fig. 7F, dark gray bars). However, while blockade of IL-2/IL-2R interactions resulted in decreased proliferation and survival of both wild-type and Ik7DN/+ during the primary stimulus (data not shown), inhibition of growth factor signaling had no effect on the ability of Ikaros-mutant T cells to produce IL-2 upon restimulation (Fig. 7F, light gray bars). These data indicate Ikaros is downstream of IL-2R signaling in the context of anergy avoidance, and that IL-2 or other growth factors that signal through the common γ-chain are not required for anergy avoidance in the absence of Ikaros function. These data demonstrate that Ikaros DNA-binding activity is required for repression of IL2 gene expression and the induction of anergy when T cells are stimulated in the absence of costimulatory signals from the CD28 or IL-2 receptors.

Discussion

Epigenetic processes such as DNA methylation and histone modification regulate T cell development (reviewed in Ref. 52), differentiation (reviewed in Ref. 53), memory (54–58), and tolerance (5, 25, 26). Ikaros, a lymphocyte-specific zinc finger DNA-binding protein required for the development of all lymphoid lineages (34, 36), is a component of several chromatin remodeling and methylation complexes, including the NURD, Sin3, and CtBP corepressors (35, 45, 46). Ikaros has been shown to negatively regulate G1-to-S phase progression in mature T cells (59), and it acts as a tumor suppressor in both T and B lymphocytes (38, 59, 60). These activities led us to test whether Ikaros might be involved in the regulation of chromatin structure and transcription at the IL2 gene.

Our studies show that Ikaros DNA-binding activity is required for the appropriate, context-dependent regulation of chromatin structure at the endogenous IL2 gene in CD4+ T cells (Fig. 4). In the presence of normal Ikaros DNA-binding activity, the nucleosomes positioned at the IL2 promoter/enhancer in quiescent T cells are hypoacetylated (Ref. 5 and Fig. 4). Likewise, signals from the TCR are not sufficient to induce acetylation and remodeling of these nucleosomes, whereas costimulation through the TCR and CD28 are sufficient to induce strong histone acetylation and chromatin remodeling at the IL2 locus. The establishment of stable histone acetylation at this locus after the return to quiescence is associated with the faster and greater expression of this gene upon Ag re-encounter (5, 61). However, in the absence of Ikaros DNA-binding activity, the IL2 promoter/enhancer exhibits TCR- and CD28-independent histone acetylation (Fig. 4) characteristic of “poised” cytokine gene loci in functional effector and memory T cells (5, 54), with hyperacetylation apparent on both histone H3 (Fig. 4) and H4 (62). These differences in acetylation are not due to large-scale differences in the density of nucleosomes positioned across the IL2 promoter, as we observe similar bulk histone H3 occupancy upstream of the IL2 transcription start site in naive, anergic, and effector T cells from both wild-type and Ikaros-mutant mice (our unpublished observations).

Therefore, the type of histone depletion that has been reported at the IL2 promoter in EL4 thymoma cells stimulated with phorbol ester (31) does not appear to be operative in our primary CD4+ T cell system.

These data suggest that Ikaros activity is important for maintaining effector genes in a closed or neutral state until the appropriate combination of signals has been achieved. In this sense, Ikaros behaves very similarly to Foxp3 in regulatory T cells, which mediates rapid and stable deacetylation of the IL2 promoter when transduced into conventional CD4+ T cells (42). Ikaros binds to several corepressor complexes, including NURD, Sin3A, Sin3B, and CtBP (35, 45, 46). Each of these complexes contain HDAC enzymes that are likely responsible for the capacity of Ikaros to maintain histone hypoacetylation at the IL2 promoter region in the absence of TCR/CD28 signals. Further study is required to identify...
the exact chromatin-remodeling complexes that are recruited to the IL2 locus. We therefore show that Ikaros DNA-binding activity in CD4+ T cells results in repression of IL2 transcription. This activity of Ikaros at the IL2 locus in mature CD4+ T cells is reminiscent of its role in the down-regulation of TdT gene expression in double-positive thymocytes (63). DNA sequences in the TdT promoter that bind Ikaros in vitro are required for silencing of TdT gene expression in developing thymocytes in response to TCR-coupled signal transduction. As with Ikaros-dependent repression of the IL2 locus, down-regulation of TdT gene expression is accompanied by the development of a closed chromatin structure, and this precedes targeting of the TdT locus to pericentromeric heterochromatin. A similar role for Ikaros has been demonstrated in the regulation of chromatin structure and repression of transcription at the IL4 locus in mast cells (64). Repression of gene transcription by Ikaros has been shown in other models to be mediated by recruitment of the NURD, Sin3A, Sin3B, or CtBP corepressor complexes to gene promoters (65). Whether these complexes are recruited to the IL2 promoter is not known, and further study is required to determine which corepressor(s) are responsible for Ikaros-mediated repression of the IL2 gene.

Our data demonstrate that Ikaros DNA-binding activity at both these sites in naive, quiescent CD4+ T cells (Fig. 6). The −17 site (GGGAGA) defined in our study falls within the TATA-1 box of the proximal IL2 promoter, and is a perfect match for only the core Ik2 consensus (GGGAGA) (44). This may explain why this site binds with lower affinity than the −203 site to Ikaros from T cell nuclear extracts in vitro (Fig. 1). We were able to detect Ikaros DNA-binding activity at both these sites in naive, quiescent CD4+ T cells (Figs. 1 and 2). These results are consistent with previous in vivo footprinting studies showing that both the TCEd/NFIL-2C element and nucleotides surrounding the transcriptional start site are occupied in unstimulated T cells, but not in non-T lineage cells (66), by factor(s) that actively contribute to repression of the IL2 gene in resting T cells (67). Our data demonstrating Ikaros binding at −203 bp and repression of IL2 gene transcription by this factor may also explain the unexpected results of previous studies in which mutations in the TCEd/NFIL-2C region led to increased IL2 promoter/reporter activity in murine CD4+ T cell clones (23).

The −203 Ikaros element physically overlaps a defined NF-κB-binding site (68), and therefore Ikaros could compete with NF-κB for binding to the TCEd/NFIL-2C region. Consistent with this, mutation of the −203 Ikaros core element (GGGAT→GGCAT), which abolishes Ikaros binding in vitro (Fig. 1), also abolishes inducible IL2 promoter activity in Jurkat cells (our unpublished observations), presumably by inhibiting binding of p65/p50 heterodimers to the TCEd/NFIL-2C element (69). However, previous studies also have shown that the IL2κB element within the TCEd/NFIL-2C region of the IL2 promoter/enhancer is bound by p50/p50 homodimers, which contribute to repression of IL2 in resting and anergic T cells (70, 71). It is not clear from our current data whether Ikaros competes or synergizes with p50/p50 homodimers at the IL2κB site. Binding of Ikaros and p50 may be mutually exclusive, with each acting as a “back-up” mechanism to ensure that the IL2 gene is not transcribed without costimulatory signals. Alternatively, p50/p50 homodimers and Ikaros may bind to the IL2κB/TCEd region at the same time, and synergize to repress IL2 transcription under conditions of suboptimal activation. Similarly, Ikaros binding to the −17 site in resting cells could compete for binding of the TATA-binding protein component of the basal transcriptional machinery to the primary TATA box. Further studies are required to determine whether competition or synergy with Rel family members or other factors represents an important mode of repressive action by Ikaros at the IL2 locus.

Our studies provide new insights into the mechanisms by which Ikaros regulates gene expression. Previous studies have suggested that direct DNA binding to gene promoters by Ikaros induces transcriptional activation (44), while recruitment of Ikaros by heterologous DNA-binding domains leads to gene repression (46). However, we show here that Ikaros binds to the endogenous IL2 promoter directly through its DNA-binding domain (Figs. 1 and 2), yet this mode of recruitment results in repression of IL2 transcription, not activation. These results demonstrate that Ikaros can mediate transcriptional repression via direct DNA binding to a gene promoter. Similarly, it has been unclear whether gene repression by Ikaros necessarily requires targeting to pericentromeric heterochromatin. Although repression of the TdT gene correlates strongly with its localization to heterochromatin in thymocytes (40, 72), Ikaros-mediated IL2 repression does not appear to correlate with heterochromatic targeting, as previous studies have shown that the majority (70–80%) of IL2 alleles in populations of wild-type naive and activated CD4+ T cells do not colocalize with pericentromeric heterochromatin (73). This suggests that targeting of genetic loci to pericentromeric heterochromatin is not a prerequisite for transcriptional repression by Ikaros.

In addition to repression of histone acetylation and transcription at the IL2 locus, our studies establish an obligate role for Ikaros in the induction of anergy in CD4+ T cells that receive TCR engagement in the absence of costimulatory or growth factor signaling. Ikaros could facilitate anergy by limiting IL-2 production during a tolerogenic stimulus, ensuring that IL-2/IL-2R signaling does not counteract the “main” biochemical mechanisms that induce and maintain anergy. Alternatively, Ikaros may represent a crucial component of the intrinsic anergic machinery that is required for repression of the IL2 gene under many or all tolerogenic situations. Our data in Fig. 7 indicate that cells lacking Ikaros function do not require signals from CD28, IL-2, or any component of the IL-2R to avoid anergy. Similarly, Ikaros has recently been defined to operate in a model of ionomycin-induced anergy (62), suggesting a general role for Ikaros in the induction of anergy via multiple mechanisms. These results together show that Ikaros is a core component of the anergic machinery.

However, our studies also show that Ikaros is not an anergy-specific factor. Studies in the ionomycin system have suggested that anergic T cells express ~2-fold more protein than naive cells, and that Ikaros preferentially binds to the IL2 promoter in anergic, but not resting T cells (62, 74). Conversely, we find that naive, effector, and anergic CD4+ T cells in our costimulatory blockade models express comparable amounts of Ikaros protein isoforms, and our sensitive, quantitative analyses of in situ Ikaros binding clearly show that Ikaros binds to the IL2 promoter/enhancer in resting CD4+ T cells. Therefore, Ikaros can mediate anergy when present at levels expressed in naive cells, suggesting that Ikaros activity may be differentially regulated at a posttranslational level by costimulatory signals from CD28 or IL-2R. For instance, Ikaros
has recently been shown to be phosphorylated in response to mitogenic signals (75), and this modification inhibits Ikaros DNA-binding activity. It is possible that Ikaros is differentially phosphorylated under effector vs anergic conditions, however, we do not find differences in total nuclear Ikaros-binding activity in anergic vs effector CD4+ T cells as measured in vitro by EMSA (our unpublished observations). Ikaros can also be subject to SUMOylation (76), which does not alter its DNA-binding activity, but rather inhibits its association with coexpressor complexes. Therefore, differential SUMOylation in anergic vs effector cells could alter the constituents of Ikaros complexes that bind to the IL2 promoter and impact the repressive capacity of Ikaros at this locus.

Our studies have identified Ikaros as an important regulator of IL2 chromatin structure and transcription that is required for the induction of T cell clonal anergy. These results suggest that Ikaros is crucial not only for T lymphocyte development, but also for the adoption of tolerant vs effector cell fates in mature CD4+ T cells during peripheral immune responses.

Acknowledgments

We thank G. Koretzky, J. Monroe, and S. Reiner for critical review of the manuscript.

Disclosures

The authors have no financial conflict of interest.

References


