The Journal of Immunology

Krüppel-Like Factor 2, a Novel Immediate-Early Transcriptional Factor, Regulates IL-2 Expression in T Lymphocyte Activation

Jinghai Wu and Jerry B. Lingrel

J Immunol 2005; 175:3060-3066; ;
doi: 10.4049/jimmunol.175.5.3060
http://www.jimmunol.org/content/175/5/3060

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Krüppel-Like Factor 2, a Novel Immediate-Early Transcriptional Factor, Regulates IL-2 Expression in T Lymphocyte Activation[1]

Jinghai Wu and Jerry B. Lingrel[2]

Ag presentation to T lymphocytes and subsequent activation are characterized by a cascade of signaling events, some of which result in the transcriptional activation of a diverse set of genes. An important example is the induction of the IL-2 gene, which is a critical event in the escalation of T cell activation. Previous studies have found that expression of Krüppel-like factor 2 (KLF2), a zinc finger transcription factor, is extinguished after T cell activation. However, the biological role of KLF2 during T cell activation is still unknown. In this study we found that KLF2 protein degradation is delayed, and KLF2 expression is up-regulated during the early stage of T cell activation in primary T cells. Within a few hours, this process is reversed, and KLF2 expression is turned off. Next, we found that the expression of KLF2 significantly increases IL-2 production 4-fold in activated T cells, resulting from activation of the IL-2 promoter. By narrowing down the 2.0-kb IL-2 promoter region, we found that the KLF2 responsive element in the IL-2 promoter is a CACCC element, the KLF consensus binding motif. Moreover, KLF2 binds to this promoter in vivo under different conditions. Our studies show that KLF2 regulates IL-2 promoter activity in the earliest stages of T cell activation, indicating that KLF2 may act as a novel immediate-early transcriptional factor to maximally prime T cell activation. ***The Journal of Immunology,*** **2005, 175: 3060–3066.**

An essential feature of the cellular immune response is the activation of T lymphocytes. T cells are activated by triggering the TCR/CD3 complex by a specific Ag, in collaboration with costimulatory and adhesion receptors. This is a complex process that involves multiple enzymes, adapters, and other transcriptional factors, which induces a cascade of metabolic events resulting in the transcriptional activation of a large number of different genes (1). Of these genes, the best characterized is the IL-2 gene, whose induction is critical for T cell activation and differentiation. The quantity of IL-2 produced is a major determinant of whether an effective response can be generated. Previous studies have indicated that several transcriptional factors, including AP-1, NFAT, NF-κB, and CD28RE/AP, are important positive regulators of IL-2 gene expression (2). Also, a CACCC binding site for specificity protein 1 and an early growth response gene-1 binding site have been recently identified immediately upstream of the distal NFAT site (3). Because many of these transcriptional factor binding sites vary from consensus sequences in other genes, it appears that these differences may in part account for the T cell-specific expression of IL-2.

Krüppel-like factor 2 (KLF2[3]; previously termed LKLF) is a member of the closely related family of the KLFs. KLF2 is highly expressed in fetal and adult lungs, heart, as well as several other organs (4, 5). It is absolutely required for normal embryogenesis and late stages of lung development (6–8). KLF2 homozygous null mice die in utero due to severe hemorrhage resulting from defects of the mature blood vessel wall (6). KLF2 does not seem to be a regulator of the initiation of blood vessel morphogenesis; however, it is active in the late stages of blood vessel wall assembly and stabilization (7). KLF2 also appears to play an important role in endothelial cell activation (9, 10).

Additionally, KLF2 plays an important role in T cell function. KLF2 is developmentally induced during the maturation of single-positive $CD4^+$ or $CD8^+$ T cells and prevents mature circulating cells from undergoing apoptosis (11, 12). Furthermore, it is required to maintain $CD4^+$ or $CD8^+$ single-positive T cells in their quiescent state (13). Also, KLF2 inhibits Jurkat T leukemia cell growth via direct up-regulation of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ (14). Intriguingly, KLF2 is rapidly extinguished after T cell activation and is re-expressed in $CD8^+$ memory T cells (11, 15), indicating that KLF2 might also play a crucial role in regulating the activation of T cells and the survival of memory T cells. However, the exact biological role of KLF2 in T cell activation is still unknown. In the studies presented in this paper, we found that KLF2 transcription and protein expression are up-regulated during the early stage of T cell activation. Moreover, the expression of KLF2 significantly increases IL-2 production, up to 4-fold in activated T cells. Our studies also suggest that KLF2 maximally regulates IL-2 promoter activity directly via a CACCC KLF2 binding site in the earlier stage of T cell activation. Our findings indicate that KLF2 may be required to maximally prime T cell activation as a novel immediate-early transcriptional factor.

Materials and Methods

Plasmid constructs

Full-length mouse KLF2 cDNA tagged at the C terminus with hemagglutinin (HA) was generated by PCR and inserted into *Bam*HI/*Hin*dIII restriction sites of vector pBK-CMV (Stratagene) as previously described (14).

Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati, College of Medicine, Cincinnati, OH 45267

Received for publication December 30, 2004. Accepted for publication June 22, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grant RO1HL57281 (to J.B.L.).

[2] Address correspondence and reprint requests to Dr. Jerry B. Lingrel, Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, OH 45267. E-mail address: jerry.lingrel@uc.edu

[3] Abbreviations used in this paper: KLF, Krüppel-like factor; ChIP, chromatin immunoprecipitation; Dox, doxycycline; HA, hemagglutinin; mt, mutant; PPARγ, peroxisomal proliferator-activated receptor γ; wt, wild type.

0022-1767/05/$02.00

Subsequently, the insert including full-length, wild-type KLF2 (KLF2-wt) with the HA tag was subcloned into the pTRE2hyg vector (BD Clontech) to generate Tet/KLF2 as a response plasmid for doxycycline (Dox) induction.

Cell line and stable cell transfection

The human Jurkat tet-on CD4$^+$ T cell line (BD Clontech) was maintained in RPMI 1640 medium supplemented with 10% Tet System Approved FBS (BD Clontech), 100 μg/ml G418, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml gentamicin. The transfection of 30 μg of Tet/KLF2-wt plasmid was conducted by electroporation of 2×10^7 cells with settings at 950 μF and 240 V, respectively. Stably transfected cells were selected by growth in 200 μg/ml hygromycin (BD Clontech) and 200 μg/ml G418 (Invitrogen Life Technologies). Clones from single cells were generated by limited dilution. Protein expression was induced by resuspending cells at a density of 10^5/ml in complete medium with Dox (1 μg/ml; Sigma-Aldrich), a derivative of tetracycline. Dox-containing medium was replaced daily during all experiments to maintain protein expression. The stable clones were individually screened for high Dox inducibility by Western blotting and named J/KLF2. Jurkat T cells (ATCC TIB-152; American Type Culture Collection) were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Preparation of human PBL

PBL were isolated from human peripheral blood by density centrifugation and depletion of adherent cells on plastic culture dishes (16). Cells were maintained in RPMI 1640 medium containing 10% FCS and 2 mM glutamine at 37°C in a 5% CO_2 atmosphere. PBL were counted in a Neubauer chamber and seeded at a cell density of 5×10^5 cells/ml into six-well plates. Cells were stimulated with PHA (5 μg/ml) at different time points (16).

RNA isolation and Northern blot analysis

RNA of human leukocytes and human heart were purchased from BD Clontech. Total RNA of Jurkat T cells and SW480 colon cancer cells were isolated with TRIzol reagent (Invitrogen Life Technologies) and subjected to Northern blot analysis. Northern blot analysis was performed as described previously (17). Briefly, an aliquot of total RNA (5 μg/lane) was size fractionated by 1% agarose gel electrophoresis and transferred to a nylon membrane. Blots were immobilized by UV cross-linking and hybridized to [α-^{32}P]dCTP-labeled cDNA probe from the 3′-untranslated region of human KLF2. The bands were detected using a Storm 860 phosphorimager (Molecular Dynamics). Human β-actin cDNA probe was used as an internal control.

Western blot analysis

Cells were lysed in 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 100 mM sodium fluoride, 10 mM $Na_4P_2O_7$, 1 μM PMSF, and 1 μM Na_3VO_4 (all chemicals from Sigma-Aldrich).The protein concentration was determined with a commercially available bicinchoninic acid kit (Pierce). Samples were fractionated by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore). After blocking, 0.5 μg/ml rabbit polyclonal Ab raised against HA (Santa Cruz Biotechnology) was incubated with the membrane. The secondary Ab, horse HRP-conjugated goat anti-rabbit (Amersham Biosciences), was used at a 1/10,000 dilution. Protein-Ab interaction was visualized by chemiluminescence detection using an ECL Western blotting system (Amersham Biosciences).

[^{3}H]thymidine incorporation

Proliferation was determined by estimating [^{3}H]thymidine incorporation into DNA. Jurkat clones (1×10^5) in 24-well dishes were incubated with control medium or medium containing Dox for 48 h. Cells were pulsed with 1 μCi/ml [^{3}H]thymidine 6 h before harvest. Disintegrations per minute were estimated by liquid scintillation counting.

ELISA

After J/KLF2-wt and Jurkat tet-on cells were treated with 1 μg/ml Dox for 24 h, cells were synchronized overnight in the medium containing 0.5% FBS. Cells were washed with RPMI 1640, adjusted to 2×10^6 cells/ml, and stimulated with PHA (1 μg/ml) and PMA (50 ng/ml) for 24 h in the medium without Dox. Cells were removed by centrifugation, and the supernatants were assayed for the presence of IL-2 using a human IL-2 ELISA kit (Pierce) according to the manufacturer's instructions.

Generation of promoter constructs and site-directed mutagenesis

A 2-kb fragment of IL-2 enhancer/promoter sequence, provided by Dr. V. Boussiotis (Harvard Medical School, Boston, MA) (18), was cloned into the pT81-Luc reporter vector. The −585 IL-2 promoter construct, was inserted into the *Sal*I/*Bgl*II site of pT81-luc. The empty vector of pT81-luc, NFAT, and NF-κB luciferase reporter constructs were gifts from Dr. D. McKean (Mayo Clinic, Rochester, NY) (19, 20). 4xAP-1 and CD28RE/AP luciferase reporters were provided by Dr. X.-F. Wang (Duke University Medical Center, Durham, NC) (21) and Dr. A. Weiss (University of California, San Francisco, CA) (22), respectively. Three additional IL-2 luciferase deletion mutants (pIL2–310, pIL2–290, and pIL2–206) were created to narrow down the potential KLF2-responsive sites. PCR was used to amply these deletion mutants and to introduce a *Kpn*I restriction site at the 5′ end, starting from IL-2 promoter region sites −310, −290, and −206, respectively, and the *Bgl*II restriction site at the 3′ end, ending in the IL-2 transcriptional start codon (forward, 5′-CGAGGTACCTTTGTGTCTC CACCC, 5′-CGAGGTACCGAGGAAAATTTGTTTC, and 5′-CGGGGT ACCATCCATTCAGTCAGTG; reverse, 5′-GATAGATCTAGGC AGCTCTTCAGC). The pIL2–310 plasmid was used as a template for generation of the distal CACCC mutation. This CACCC binding site was mutated using the QuikChange mutagenesis kit according to the manufacturer's recommendations (Stratagene). This mutation changed the wt sequence 5′-TTTGTGTCTCCACCCCAAAGAGGAAA-3′ to 5′-TTTGT GTCTCCAGTGCAAA GAGGAAA-3′. The underlined sequences mean the mutated sequences.

Promoter-reporter assays

For transient transfection in suspension cells, Jurkat clones were washed with cold RPMI 1640 medium, resuspended in 250 μl of fresh medium at 2×10^7/ml, and electroporated at room temperature with 40 μg of plasmid DNA using a Gene Pulser (Bio-Rad) with settings of 240 V and 950 μF. The cells were then resuspended in fresh medium and maintained for 1 h at 37°C. Dox was added to induce KLF2 expression. Cell lysates were prepared 48 h later, and luciferase activity was measured using a luciferase assay kit (Promega) and a Monolight 3010 luminometer (BD Pharmingen). β-Galactosidase activity was measured with a commercially available kit (Promega). The promoter activity of each construct was expressed as the ratio of luciferase/β-galactoside activity. All transfections were performed in triplicate in three independent experiments.

Chromatin immunoprecipitation (ChIP) assays)

J/KLF2 cells were treated with Dox and without Dox, respectively, for 48 h and stimulated with or without PHA and PMA. Native protein-DNA complex were cross-linked by 1% formaldehyde for 10 min. The ChIP assays were performed by modification of a previously reported procedure (23). Briefly, equal aliquots of isolated chromatin DNA were subjected to immunoprecipitation with a rabbit anti-HA Ab or rabbit IgG control. The DNA fragments associated with specific immunoprecipitates or with the control IgG were purified and used as templates for the radioactive PCR to amplify the IL-2 promoter sequences containing the CACCC/KLF2 binding site. The primers used were 5′-GTCCACCACAATATGCTATTCAC and 3′-CCACACTTAGGTGATAGCTC. In addition, an IL-2 promoter region that is 1.6 kb upstream from the CACCC/KLF2 binding site was used as a negative control to confirm the KLF2 binding specificity. The primers used are as follows: 5′-CATTCCTGCCTATGCACCCTC and 3′-CTGGT GAT GGTGATTGCTG.

Results

Jurkat Th cells as a study model of KLF2

The Jurkat CD4$^+$ T cell line has been widely used in the studies of T cell activation and IL-2 production. Because it has been shown previously that KLF2 protein is not produced in Jurkat T cells (13), and that mRNA levels of KLF2 were not expressed in Jurkat cells (14), we chose this cell line as a model to study the role of KLF2 during T cell activation. Initially, we confirmed that the expression level of mRNA of KLF2 was very low in Jurkat cells compared with normal human leukocytes, human heart tissue, and SW 480 human colon cancer cells (Fig. 1*A*). Thus, Jurkat cells are an excellent model to study the biological significance of KLF2 in lymphocytes by ectopic re-expression of this transcription factor. To study the role of re-expression of KLF2 in human T cells, we used a Dox-inducible system in Jurkat T cells (13, 14). As shown in Fig. 1*B*, before Dox treatment, KLF2 expression was undetectable in

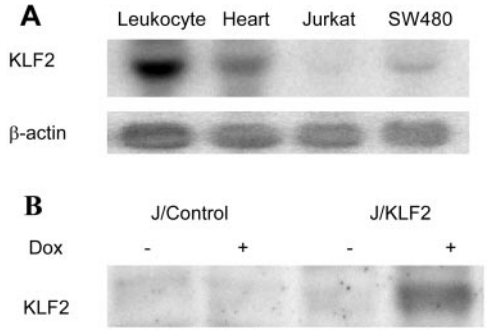

FIGURE 1. *A*, Expression of KLF2 mRNA in Jurkat Th cells and control tissues. Five micrograms of total RNA per sample was subjected to Northern blot analysis. The blot represents RNA hybridized to a radiolabeled cDNA probe from the 3′-untranslated region of human KLF2. In the *bottom* blot, equal loading was confirmed by reprobing with β-actin. *B*, Expression of KLF2 in Jurkat T cells with the use of a Dox-inducible system. Immunoblot analysis of KLF2 expression with anti-HA Ab in the 25 μg of cellular extracts of Jurkat clones 24 h after treatment with Dox-free control medium (−) or medium containing Dox (+).

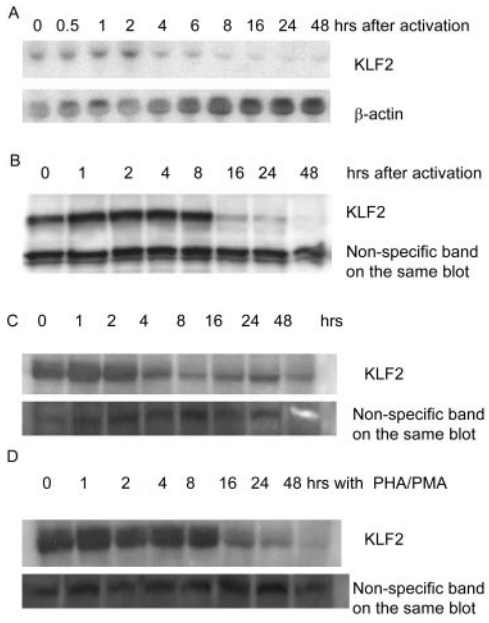

FIGURE 2. KLF2 expression levels are up-regulated during the early stage of T cell activation. KLF2 expression patterns were examined in human normal PBL cells upon activation (*A*, Northern blot; *B*, Western blot). J/KLF2 cells were incubated in the medium containing Dox (1 μg/ml) for 24 h; cells were washed with RMPI 1640 and cultured without PHA/PMA (*C*) or PHA/PMA (*D*) for various times. Cellular extracts (50 μg) were fractioned by SDS gel. Equal loading of cellular extracts was confirmed by equivalent amounts of a high molecular size band on the same blot.

the KLF2-transfected clone (J/KLF2) and control Jurkat cells. In contrast, after the addition of Dox, KLF2 was expressed at high levels only in J/KLF2.

Loss of KLF2 is delayed during the early stage of T cell activation and accelerated during the late stage of T cell activation

It has been reported that both KLF2 mRNA and protein levels are rapidly extinguished after T cell activation (11, 15). However, the exact time course of KLF2 degradation has not been studied. We studied the KLF2 expression pattern in normal PBL (16). As shown in Fig. 2, *A* and *B*, KLF2 expression was up-regulated as early as 30 min to 1 h after activation at the transcription and translation levels, respectively. These data demonstrated that the initial activation enhances KLF2 expression before promoting a decrease. In the J/KLF2 clone, we initially induced KLF2 expression by removing Dox after 24 h and then stimulated T cells with PHA and PMA, which are known to efficiently activate T cells through TCR and protein kinase C pathways (16, 24, 25). As shown in Fig. 2*C*, in the absence of PHA/PMA stimulation, induced KLF2 began to be lost by 4 h, a process continued over the next 48 h. If KLF2 is negatively regulated during T cell activation, as suggested in previous reports (11, 15), loss of KLF2 after treatment with PHA/PMA should be faster than that in cells not treated with PHA/PMA. However, with stimulation by PHA/PMA, high expression of KLF2 was sustained until 8 h and then rapidly extinguished to a very low level (Fig. 2*D*). This time course and expression pattern are consistent with primary PBLC activation, suggesting that KLF2 may have some biological role in the early stage of T cell activation.

KLF2 inhibits DNA synthesis in activated T cells

We previously have shown that KLF2 inhibited new DNA synthesis in Jurkat cells (14), suggesting that KLF2 has an antiproliferative effect. Therefore, it was important to test whether KLF2 still has an antiproliferative role in activated T cells. Cellular proliferation was measured by [^{3}H]thymidine incorporation in J/control and J/KLF2 cells. As shown in Fig. 3, Dox itself does not affect thymidine incorporation in J/control cells after treatment with PHA/PMA similar to that in cells not treated with PHA/PMA. However, KLF2 induced by Dox still inhibited new DNA synthesis to ~70% in J/KLF2 after treatment with PHA/PMA, suggesting that KLF2 also inhibits cell proliferation during activation.

KLF2 transcriptional activity is up-regulated during the very early stage of T cell activation, and KLF2 regulates IL-2 secretion in activated Jurkat Th cells

Because it is known that protein expression of KLF2 is sustained during the early stage of activation, we also studied the transcriptional activity of the KLF2 promoter. Transient transfection of KLF2 promoter luciferase reporter constructs indicated that KLF2 transcription is up-regulated as early as 15 min after T cell activation, reaching a maximum at 30 min of T cell activation, then quickly decreasing to levels even lower than those before activation (Fig. 4*A*). We also used a peroxisomal proliferator-activated receptor γ (PPARγ) promoter as a control. As shown in Fig. 4*A*, PPARγ transcriptional activity was not increased during T cell activation, emphasizing the specificity of the KLF2 response. These data reveal that the KLF2 promoter is activated at a very early stage of T cell activation and are consistent with the KLF2 protein degradation seen after T cell activation. Therefore, KLF2 may act as an immediate-early transcriptional factor in T cell activation.

Jurkat Th cells have been widely used as a model for the regulation of T cell activation, especially for IL-2 gene regulation, an important event in T cell activation and differentiation. Consistent with several reports, in the absence of PHA/PMA, control Jurkat T cells and J/KLF2 cells did not produce measurable amounts of IL-2. After stimulation, IL-2 secretion in both control and J/KLF2 cells significantly increased to a similar extent. However, cells with Dox-induced KLF2 expression produced much more IL-2, up to 4-fold more compared with that in Dox-treated control cells (Fig. 4*B*). These data reveal that KLF2 is a novel regulator of IL-2

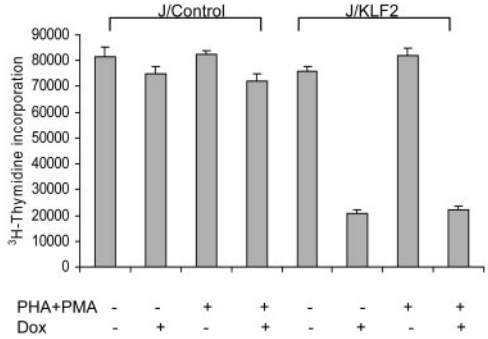

FIGURE 3. KLF2 inhibits DNA synthesis of activated Jurkat T cells. Jurkat control (J/control) and Jurkat/KLF2 (J/KLF2) clones were incubated in medium with or without Dox treatment. PHA (1 μg/ml) and PMA (5 ng/ml) were added to medium to stimulate T cells for 24 h. Each group was studied in triplicate (three wells). [^{3}H]thymidine incorporation was assessed for the last 6 h of a 48-h total culture period.

production. Because KLF2 is mainly expressed in resting T lymphocytes that do not synthesize IL-2, and IL-2 is not expressed in unactivated J/KLF2 cells producing KLF2, we propose that KLF2 is not sufficient for IL-2 expression, but is involved in the regulation of IL-2 production shortly after cellular activation. This may explain the increased KLF2 expression immediately after activation.

KLF2 maximally regulates IL-2 promoter activity at an early stage of T cell activation

To study the mechanism of KLF2 in the regulation of the IL-2 synthesis, we determined whether the IL-2 promoter responded to KLF2 in transient transfection assays. Consistent with previous ELISA results, there is no IL-2 promoter activity in unstimulated T cells. After the addition of PHA/PMA, IL-2 expression was increased, and the presence of KLF2 increased IL-2 expression by ~5-fold, suggesting that the up-regulation of IL-2 promoter activity by KLF2 contributed to the enhancement of IL-2 secretion (Fig. 5*A*). As is shown in Fig. 5*B*, 2 h after T cell activation, KLF2 maximally enhanced the induction of IL-2 promoter activity, suggesting that KLF2 may enhance IL-2 production by responding to an immediate-early signal transduction pathway.

KLF2 does not affect NF-κB, NFAT, AP-1, or CD28RE/AP-1 signal transduction pathways

Several transcriptional factors, including NF-κB, NFAT, AP-1, and CD28RE/AP-1, are known to be important positive regulators of activation-specific T cell gene expression such as IL-2. We hypothesized that KLF2 might also regulate classical signal transduction pathways. However, transient transfection assays revealed that KLF2 did not significantly alter the promoter reporter activities of these factors (Fig. 6*A*). This indicates that KLF2 must regulate IL-2 production by an uncharacterized pathway, rather than one of the common pathways.

A KLF2-responsive element is located in a CACCC site of the IL-2 promoter

Because several suspected transduction pathways were not involved in the KLF2 enhancement of IL-2 production, we searched for the KLF2-responsive element by narrowing down the 2.0-kb IL-2 promoter. As shown in Fig. 6*C*, even after 1.5 kb of IL-2 promoter was removed, KLF2 was still able to enhance the activity of an IL-2–585 promoter. Similar high induction was also observed in a shorter IL-2–310 construct, suggesting that the KLF2-

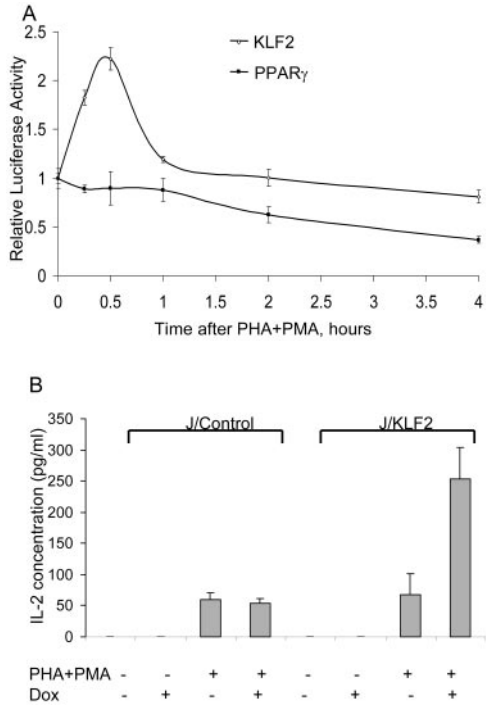

FIGURE 4. *A*, KLF2 transcription is up-regulated during the very early stage of T cell activation. Wild-type Jurkat T cells were transfected with a minimal KLF2 promoter luciferase reporter plasmid, a PPARγ promoter (−213 to start codon) luciferase reporter, and β-galactosidase as an internal control for 40 h. Cells were then stimulated by PHA/PMA for 15 min, 30 min, 1 h, 2 h, and 4 h. Each group was studied in triplicate (three wells). The fold induction of KLF2 promoter activity was measured by correction of relative luciferase activity of KLF2 promoter over empty vector. *B*, KLF2 regulates IL-2 production in PHA/PMA-activated T cells. J/control and J/KLF2 cells were incubated in medium with or without Dox treatment for 24 h. PHA (1 μg/ml) and PMA (5 ng/ml) were added to medium to stimulate T cell activation after the first 24 h of Dox treatment. Each group was studied in triplicate (three wells). After 48 h, supernatants were collected for ELISA. Every sample was assayed in triplicate.

responsive element is located in the well-characterized 300-bp region of the IL-2 promoter as shown in Fig. 6*D*. This region contains several classic transduction pathway-responsive elements that were excluded by the data in Fig. 6*A*, such as NF-κB, NFAT, AP-1, and CD28RE/AP. Through sequential deletion of the IL-2 promoter fragments, such as IL-2–206 and IL-2–290, we found that deletion of the −310 to −290 region completely abrogated the induction of IL-2 by KLF2 (Fig. 6*C*). This indicates that the KLF2-responsive element may be located in this region. In this 20-bp region, we found a KLF binding site candidate sequence, CACCC, previously reported as a specificity protein 1/early growth response gene-1 binding site. To test whether this CACCC sequence in the 20-bp region of IL-2 promoter is important for KLF2 regulation, we mutated this sequence to CATGT. This mutant luciferase reporter vector was designated IL-2–310mt, as shown in Fig. 6*B*. Subsequent transient transfection showed that this IL-2–310mt failed to respond to KLF2 induction. This confirmed that this CACCC site is the KLF2-responsive element.

KLF2 binds to the CACCC/KLF2 binding site in the IL-2 promoter in vivo

With regard to the mechanism of KLF2 on regulation of this CACCC/KLF2-responsive element, there are two possibilities: either KLF2 directly transactivates IL-2 promoter activity, or KLF2

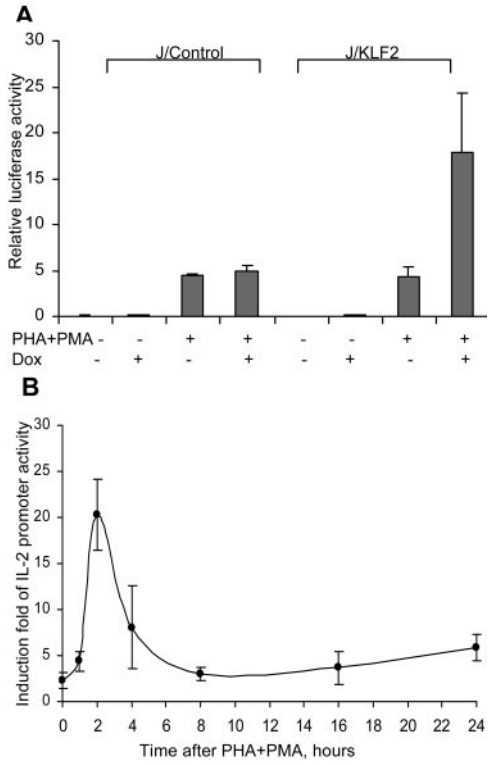

FIGURE 5. KLF2 up-regulates IL-2 promoter activity in activated T cells (*A*) and has a maximal regulation at the early stage of T cell activation (*B*). Transfection of 20 mg/ml IL-2 promoter (2.0 kb) enhancer/promoter luciferase reporter plasmid and 2 mg/ml β-galactosidase internal control was conducted by electroporation. Every sample was assayed in triplicate.

may indirectly regulate IL-2 expression through other signal transduction pathways. To verify whether KLF2 binds to this CACCC/KLF2-responsive element, ChIP assays were performed. As shown in Fig. 6*E*, *I*, Dox-induced KLF2 does not bind to the CACCC/KLF2 site in nonactivated cells. However, Dox-induced HA-tagged KLF2 binds to the IL-2 promoter upon activation (Fig. 6*E*, *III*), indicating that KLF2 directly regulates IL-2 expression. To determine whether the binding shown in Fig. 6*E*, *III*, is simply due to nonspecific binding of overexpressed KLF2, we performed a ChIP assay using a IL-2 promoter region that is 1.6 kb upstream from the CACCC/KLF2 binding site to constitute a negative control. As shown in Fig. 6*E*, *IV*, Dox-induced KLF2 does not bind to the negative control region upon activation, indicating that KLF2 specifically binds to this CACCC/KLF2 site.

Discussion

These findings show for the first time that IL-2 is a target of KLF2 and, in the process, underlies the importance of KLF2 regulation to T cell activation. This study provides a novel and clear mechanism by which KLF2 regulates IL-2 expression and indicates that KLF2 may act as a novel, immediate-early transcriptional factor to maximally prime T cell activation.

Our data also provide a level of comparison between KLF2 expression levels in different human tissues. KLF2 is expressed at high levels in the lung, vasculature, and lymphoid tissues during mouse development. Also, high levels of KLF2 expression in mouse $CD4^+$, $CD8^+$ single-positive thymocytes and splenocytes was demonstrated by Northern blot analysis (4, 8, 11). In addition, human KLF2 expression is detected in lung, heart, and skeletal muscle (5). It is interesting to note that KLF2 is expressed in similar tissues in human and mouse; however, the relative abundance of expression between the two species differs. Although lung is the predominant site of KLF2 expression in mouse, its relative expression level is higher in human heart than in human lung. Our results for the first time show that human KLF2 is expressed at even higher levels in leukocytes than in any other tissues, which may account for the specific biological role of KLF2 related to the hemopoietic/immune system.

KLF2 is required to program and maintain the quiescent phenotype in mature, single-positive T cells (11, 13). Also, KLF2 mRNA and protein levels are extinguished after treatment with anti-CD3 (11). Because, as noted in previous reports, this treatment could lead to induction of anergy rather than full activation of T cells (15, 26, 27), it raises the issue of whether KLF2 is really involved in human T cell activation, and whether it plays a positive or a negative regulatory role in this process. In this context, we addressed these questions. 1) Is KLF2 expression extinguished in fully activated $CD4^+$ T cells? 2) What biological role does KLF2 have during early T cell activation? 3) What are the target genes of KLF2 during early T cell activation?

Although KLF2 protein levels of $CD8^+$ T cells decreased 24 h after activation (15), it is still necessary to understand whether similar processes and outcomes occur in $CD4^+$ T cells, which are also a major source of critical cytokines such as IL-2. KLF2 expression patterns in primary human PBL show that KLF2 is not only extinguished upon activation, but, more intriguingly, KLF2 is up-regulated during the early stage of cell activation. These findings are also confirmed by our studies of both KLF2 promoter activity and protein degradation, followed by full T cell activation, in Jurkat $CD4^+$ T cells. These data revealed that KLF2 has a novel biological role in typical T cell activation.

The mechanism of KLF2 degradation after T cell activation is currently under investigation. Previous work indicates KLF2 degradation is mediated by a protein kinase C-dependent pathway (11), and that degradation is preceded by alteration in the electrophoretic mobility of the protein, suggesting that phosphorylation or ubiquitination might regulate the stability of KLF2 in T cells upon activation. Additionally, we have shown that WW domain-containing protein 1, an E3-ubiquitin ligase, interacts with KLF2 in vivo and mediates both polyubiquitination and proteasomal degradation of KLF2 (28). A similar mechanism is likely to degrade KLF2 in this system.

Interestingly, we found that KLF2 enhanced IL-2 secretion >4-fold in activated T cells. IL-2 is an essential factor required for the progression of newly activated T cells, and the quantity of IL-2 produced is a major determinant of whether an effective response can be generated. Moreover, inadequate IL-2 synthesis can lead to T cell death or induction of a state of unresponsiveness known as clonal anergy. The IL-2 gene is not actively transcribed in resting T cells or in Jurkat T cells, and the transcription of IL-2 can be detected as early as 40 min after activation of Jurkat T cells, leading to peak levels of mRNA at ~6 h, suggesting that T cell activation-related immediate-early transcriptional factors are involved in transactivation of the IL-2 gene (29–31). The NFAT complex, an early regulator of T cell activation, has been shown to appear 10–25 min before activation of the IL-2 gene (32). The increase in KLF2 expression after T cell activation also fits this pattern.

Our data show that transactivation of the KLF2 promoter is increased as early as 15 min after T cell activation, and KLF2 protein degradation is delayed until 8 h after T cell activation, suggesting that KLF2 is a novel, immediate-early transcriptional factor in T cell activation. In addition, KLF2 regulates IL-2 secretion as a positive transcriptional factor. The maximal up-regulation of the IL-2 promoter appears ~2 h after T cell activation, also indicating that KLF2 acts as an early transcriptional factor in the

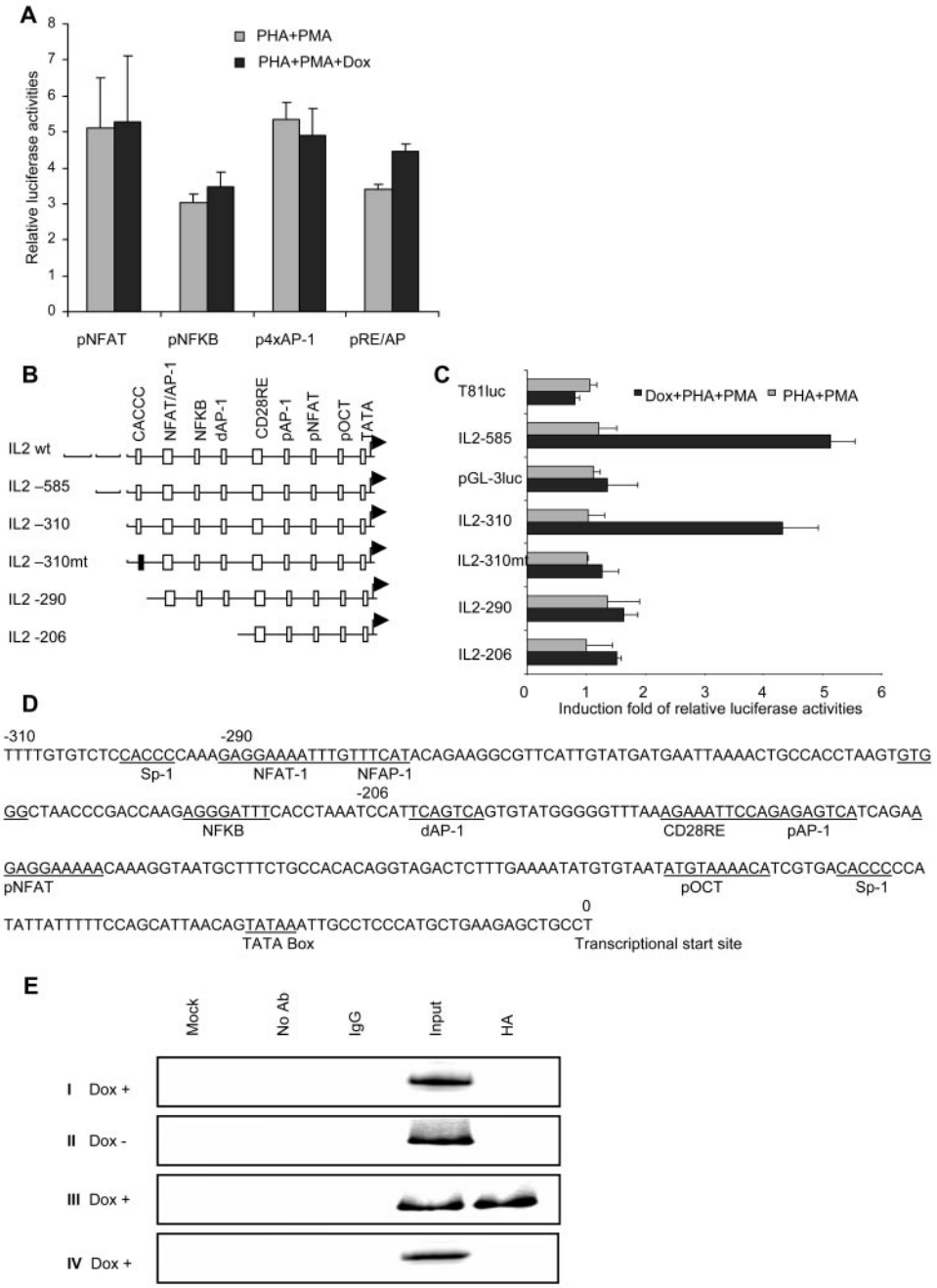

FIGURE 6. KLF2 directly regulates IL-2 promoter activities via a CACCC KLF binding site. *A*, KLF2 does not affect NF-κB, NFAT, AP-1, or CD28RE/AP-1 signal transduction pathways. NF-κB, NFAT, AP-1, and CD28RE/AP luciferase constructs were transiently transfected in J/KLF2 cells. Cells were activated by PHA/PMA without or with Dox treatment. Lysates were collected to measure the relative luciferase activities. *B*, PCR fragments of the IL-2 promoter region (relative to the transcriptional start site) were subcloned into pGL-3 luciferase reporter construct. *C*, A functional KLF2-inducible element was localized in the 5′-flanking CACCC site of the IL-2 promoter. Each plasmid along with the β-galactosidase internal control vector was transfected into J/KLF2 cells, and transiently transfected cells were stimulated by PHA/PMA without or with Dox. The fold relative luciferase activity is the proportion of Dox treatment to no Dox treatment. *D*, IL-2 promoter sequence located between −310 and 0 bp. The CACCC KLF binding site, NFAT, NF-κB, AP-1, and OCT sites are indicated by underlining and are shown below the sequence. The TATA box is also indicated by underlining. *E*, ChIP assays indicate KLF2 binding to the IL-2 promoter. ChIP assays were conducted as described in *Materials and Methods* using extracts from J/KLF2 under different conditions. *I*, Dox treatment in nonactivated cells; *II*, without treatment of Dox in the presence of activation; *III*, Dox treatment in the presence of activation; *IV*, Dox treatment in the presence of activation and using a negative control primer set that is 1.6 kb away from the CACCC/KLF2 site.

modulation of IL-2. Previous studies have shown that KLF2, a target gene of platelet-derived growth factor, is induced as early as 30 min after activation with a peak 1 h after treatment with platelet-derived growth factor-BB and then is rapidly extinguished (33). Moreover, in human cells, KLF2 is also induced 5-fold, with a peak at ~4 h, by laminar shear stress (10). Therefore, in agreement with the findings in other cell types, we propose that KLF2 is an immediate-early responsive transcription factor.

Our studies provide insight into the transcriptional basis for the ability of KLF2 to induce IL-2 expression. Previous studies indicated that ~300 bp of the IL-2 promoter are sufficient to confer cell-specific inducible expression to reporter gene constructs (34). This is consistent with our findings of the responsive element of KLF2 in this region. Within these 300 bp, several classic transcriptional factor-binding sites have been identified as positive regulatory elements in T cells, including proximal and distal NFAT, AP-1, NF-κB, and CD28RE/AP. However, our results exclude the role of KLF2 on these transcription factor-binding sites, indicating that KLF2 must have a unique regulation pathway. We found that a CACCC/KLF2 binding site occurred immediately upstream of the distal NFAT site in the KLF2-responsive motif is crucial for the inductive effects of KLF2 as shown by site mutagenesis. Coupled with ChIP assays, our data indicate that KLF2 binds to the IL-2 promoter upon T cell activation, indicating that KLF2 directly regulates IL-2 expression.

We have already shown that KLF2 inhibits new DNA synthesis in activated Jurkat cells. Additionally, KLF2 increases IL-2 expression. Similar results were shown with FK506-binding protein-associated protein FAP48, which not only inhibits Jurkat T cell proliferation, but also increases IL-2 synthesis in PHA/PMA-activated Jurkat T cells (35). KLF2 appears to act similarly to FK506-binding protein in T cell activation.

In conclusion, our findings suggest that KLF2 is a novel early responsive transcriptional factor in T cell activation and enhances the secretion of IL-2 in activated T cells. Furthermore, these studies emphasize the importance of KLF2 in the activation of T lymphocytes. Such activation is critical in the mammalian immune response to pathogens and tumor cells as well as important effectors of allergies, transplant rejection, and autoimmunity.

Acknowledgments

We thank Drs. Vicki Boussiotis, David McKean, Xiao-Fan Wang, and Arthur Weiss for kindly providing luciferase reporter constructs, respectively, and Dr. Jeffrey Leiden for anti-KLF2 Ab.

Disclosures

The authors have no financial conflict of interest.

References

1. Weiss, A., and D. R. Littman. 1994. Signal transduction by lymphocyte antigen receptors. *Cell* 76: 263–274.
2. Taniguchi, T., and Y. Minami. 1993. The IL-2/IL-2 receptor system: a current overview. *Cell* 73: 5–8.
3. Skerka, C., E. L. Decker, and P. F. Zipfel. 1995. A regulatory element in the human interleukin 2 gene promoter is a binding site for the zinc finger proteins Sp1 and EGR-1. *J. Biol. Chem.* 270: 22500–22506.
4. Anderson, K. P., C. B. Kern, S. C. Crable, and J. B. Lingrel. 1995. Isolation of a gene encoding a functional zinc finger protein homologous to erythroid Kruppel-like factor: identification of a new multigene family. *Mol. Cell. Biol.* 15: 5957–5965.
5. Wani, M. A., M. D. Conkright, S. Jeffries, M. J. Hughes, and J. B. Lingrel. 1999. cDNA isolation, genomic structure, regulation, and chromosomal localization of human lung Kruppel-like factor. *Genomics* 60: 78–86.
6. Kuo, C. T., M. L. Veselits, K. P. Barton, M. M. Lu, C. Clendenin, and J. M. Leiden. 1997. The LKLF transcription factor is required for normal tunica media formation and blood vessel stabilization during murine embryogenesis. *Genes Dev.* 11: 2996–3006.
7. Wani, M. A., R. T. Means, Jr., and J. B. Lingrel. 1998. Loss of LKLF function results in embryonic lethality in mice. *Transgenic Res.* 7: 229–238.
8. Wani, M. A., S. E. Wert, and J. B. Lingrel. 1999. Lung Kruppel-like factor, a zinc finger transcription factor, is essential for normal lung development. *J. Biol. Chem.* 274: 21180–21185.
9. Huddleson, J., S. Srinivasan, N. Ahmad, and J. B. Lingrel. 2004. Fluid shear stress induces endothelial KLF2 gene expression through a defined promoter region. *J. Biol. Chem.* 385: 723–729.
10. SenBanerjee, S., Z. Lin, G. B. Atkins, D. M. Greif, R. M. Rao, A. Kumar, M. W. Feinberg, Z. Chen, D. I. Simon, F. W. Luscinskas, et al. 2004. KLF2 is a novel transcriptional regulator of endothelial proinflammatory activation. *J. Exp. Med.* 199: 1305–1315.
11. Kuo, C. T., M. L. Veselits, and J. M. Leiden. 1997. LKLF: A transcriptional regulator of single-positive T cell quiescence and survival. *Science* 277: 1986–1990.
12. Kuo, C. T., M. L. Veselits, and J. M. Leiden. 1997. LKLF and FasL expression: correction and clarification. *Science* 278: 788–789.
13. Buckley, A. F., C. T. Kuo, and J. M. Leiden. 2000. Transcription factor LKLF is sufficient to program T cell quiescence via a c-Myc-dependent pathway. *Nat. Immunol.* 2: 698–704.
14. Wu, J., and J. B. Lingrel. 2004. KLF2 inhibits Jurkat T leukemia cell growth via direct upregulation of cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$. *Oncogene* 23: 8088–8096.
15. Schober, S. L., C. T. Kuo, K. S. Schluns, L. Lefrancois, J. M. Leiden, and S. C. Jameson. 1999. Expression of the transcription factor lung Kruppel-like factor is regulated by cytokines and correlates with survival of memory T cells in vitro and in vivo. *J. Immunol.* 163: 3662–3667.
16. Gebert, B., W. Fischer, E. Weiss, R. Hoffmann, and R. Haas. 2003. Helicobacter pylori vacuolating cytotoxin inhibits T lymphocyte activation. *Science* 301: 1099–1012.
17. Wu, J., A. Utani, H. Endo, and H. Shinkai. 2001. Deficiency of the decorin core protein in the variant form of Ehlers-Danlos syndrome with chronic skin ulcer. *J. Dermatol. Sci.* 27: 95–103.
18. Tzachanis, D., G. J. Freeman, N. Hirano, A. A. van Puijenbroek, M. W. Delfs, A. Berezovskaya, L. M. Nadler, and V. A. Boussiotis. 2001. Tob is a negative regulator of activation that is expressed in anergic and quiescent T cells. *Nat. Immunol.* 2: 1174–1182.
19. Hedin, K. E., M. P. Bell, K. R. Kalli, C. J. Huntoon, B. M. Sharp, and D. J. McKean. 1997. δ-Opioid receptors expressed by Jurkat T cells enhance IL-2 secretion by increasing AP-1 complexes and activity of the NF-AT/AP-1-binding promoter element. *J. Immunol.* 159: 5431–5440.
20. Kalli, K., C. Huntoon, M. Bell, and D. J. McKean. 1998. Mechanism responsible for T-cell antigen receptor- and CD28- or interleukin 1 (IL-1) receptor-initiated regulation of IL-2 gene expression by NF-κB. *Mol. Cell. Biol.* 18: 3140–3148.
21. Yingling, J. M., M. B. Datto, C. Wong, J. P. Frederick, N. T. Liberati, and X. F. Wang. 1997. Tumor suppressor Smad4 is a transforming growth factor β-inducible DNA binding protein. *Mol. Cell. Biol.* 17: 7019–7028.
22. Weiss, A., J. Imboden, D. Shoback, and J. Stobo. 1984. Role of T3 surface molecules in human T-cell activation: T3-dependent activation results in an increase in cytoplasmic free calcium. *Proc. Natl. Acad. Sci. USA* 81: 4169–4173.
23. Ahmad, N., and J. B. Lingrel. 2005. Kruppel-like factor2 transcriptional regulation involves heterogeneous nuclear ribonucleoproteins and acetyltransferases. *Biochemistry* 44: 6276–6285.
24. Jenkins, M. K., C. A. Chen, G. Jung, D. L. Mueller, and R. H. Schwartz. 1990. Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody. *J. Immunol.* 144: 16–22.
25. Sosinowski, T., A. Pandey, V. M. Dixit, and A. Weiss. 2000. Src-like adaptor protein (SLAP) is a negative regulator of T cell receptor signaling. *J. Exp. Med.* 191: 463–474.
26. Jenkins, M. K., A. Khoruts, E. Ingulli, D. L. Mueller, S. J. McSorley, R. L. Reinhardt, A. Itano, and K. A. Pape. 2001. In vivo activation of antigen-specific CD4 T cells. *Annu. Rev. Immunol.* 19: 23–45.
27. Schwartz, R. H. 2003. T cell anergy. *Annu. Rev. Immunol.* 21: 305–334.
28. Zhang, X., S. V. Srinivasan, and J. B. Lingrel. 2004. WWP1-dependent ubiquitination and degradation of the lung Kruppel-like factor, KLF2. *Biochem. Biophys. Res. Commun.* 316: 139–148.
29. Abraham, R. T., and A. Weiss. 2004. Jurkat T cells and development of the T-cell receptor signalling paradigm. *Nat. Rev. Immunol.* 4: 301–308.
30. Crabtree, G. R. 1989. Contingent genetic regulatory events in T lymphocyte activation. *Science* 243: 355–361.
31. Hughes, C. C., C. O. Savage, and J. S. Pober. 1990. Endothelial cells augment T cell interleukin 2 production by a contact-dependent mechanism involving CD2/LFA-3 interaction. *J. Exp. Med.* 171: 1453–1467.
32. Shaw, J. P., P. J. Utz, D. B. Durand, J. J. Toole, E. A. Emmel, and G. R. Crabtree. 1988. Identification of a putative regulator of early T cell activation genes. *Science* 241: 202–205.
33. Chen, W. V., J. Delrow, P. D. Corrin, J. P. Frazier, and P. Soriano. 2004. Identification and validation of PDGF transcriptional targets by microarray-coupled gene-trap mutagenesis. *Nat. Genet.* 36: 304–312.
34. Durand, D. B., J. P. Shaw, M. R. Bush, R. E. Replogle, R. Belagaje, and G. R. Crabtree. 1988. Characterization of antigen receptor response elements within the interleukin-2 enhancer. *Mol. Cell. Biol.* 8: 1715–1724.
35. Krummrei, U., E. E. Baulieu, and B. Chambraud. 2003. The FKBP-associated protein FAP48 is an antiproliferative molecule and a player in T cell activation that increases IL2 synthesis. *Proc. Natl. Acad. Sci. USA* 100: 2444–2449.