REGULATION OF T LYMPHOCYTE PROLIFERATION

Interleukin 2-mediated Induction of c-*myb* Gene Expression Is Dependent on T Lymphocyte Activation State

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The interaction of IL-2 with the IL-2-R plays a central role in the regulation of T lymphocyte proliferation (1). Triggering of the TCR leads to the synthesis of IL-2 (in certain T lymphocyte subsets) and to the induction of high affinity IL-2-Rs in antigen-activated T lymphocytes (2–4). It is the binding of IL-2 to the high affinity IL-2-R (and the subsequent internalization of this complex) that leads to the cascade of events resulting in T lymphocyte proliferation (5, 6).

While the role of IL-2/IL-2-R interaction in driving T lymphocytes into the cell cycle is well established, the factors that control the magnitude and duration of T lymphocyte proliferation in response to antigen are less well understood. Early evidence favored the view that IL-2 availability was the sole regulator of T lymphocyte proliferation (1, 4). Later studies on the kinetics of IL-2-R expression revealed that IL-2-Rs were only transiently expressed on activated T lymphocytes and that the level of cell surface IL-2-Rs declined as a function of time after activation (7, 8). This evidence suggested a second site of hormonal control of T lymphocyte proliferation, i.e., level of expression of the hormone (IL-2) receptor. Accordingly, antigen would then serve to trigger T lymphocyte expansion by engaging the TCR on quiescent T lymphocytes and thereby stimulate a burst of IL-2-R expression. More recently, a third possible site for the regulation of T lymphocyte proliferation has been suggested from our observations on the time course of high affinity IL-2-R expression in antigen-regulated murine T lymphocyte clones. This analysis revealed that T lymphocytes that have undergone a cycle of antigen-driven proliferation (6–10 divisions) were refractory to the proliferative stimulus of IL-2 in spite of high levels of high affinity IL-2-Rs (9). The high affinity IL-2-Rs on these quiescent refractory T lymphocytes are functional by several criteria, e.g., internalization of IL-2/IL-2-R complexes, upregulation of expression of the IL-2-R p55 gene, and protein expression. Engagement of the T lymphocyte antigen receptor on these quiescent T lymphocytes rapidly rendered the cells sensitive to the proliferative stimulus of IL-2 (9, 10). These findings implied that an intracellular regulator (or family of regulators)

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in T lymphocytes may also play a central role in the control of T lymphocyte activation/proliferation.

In this report we have examined the expression of a panel of genes in T lymphocyte clones during the IL-2-sensitive (activated) and IL-2-insensitive (quiescent) phase of their activation cycle. We have found that the c-*myb* gene is transcribed as a result of the IL-2/IL-2-R interaction in activated (day 1–3 after antigenic stimulation) but not quiescent (day 9–14 after stimulation) T lymphocytes. Therefore, the expression of the proto-oncogene c-*myb* directly correlates with the state of activation of T lymphocytes and the sensitivity of these cells to the IL-2-driven proliferative stimulus. The role of c-myb as a critical regulator of proliferation was confirmed in studies with c-myb antisense oligodeoxynucleotide. We also provide evidence for the existence of distinct IL-2-R-dependent and TCR-dependent pathways of c-*myb* expression.

Materials and Methods

Mice. (BALB/cJ × C57B6/6J)F_1 (H-2^d × H-2^b) ($CB6F_1$) mice were purchased from The Jackson Laboratory, Bar Harbor, ME.

Viruses. Influenza virus strain A/JAPAN/305/57 (H2N2) was grown in the allantoic cavities of 10-d-old chick embryos. Infectious allantoic fluid was harvested 2 d later, aliquoted, and stored at −70°C.

T Lymphocyte Clones. The murine T lymphocyte clones were established as described in detail elsewhere (11). Briefly, the clones used in this study were derived from the spleen of A/JAP/57 influenza virus-immune $CB6F_1$ mice by limiting dilution in the presence of antigen (irradiated, A/JAP/57-infected, syngeneic spleen cells), plus a source of IL-2 (supernatant from Con A-stimulated rat spleen cells) in Iscove's modified Dulbecco's medium (Gibco Laboratories, Grand Island, NY), plus 10% FCS (Sterile Systems, Logan, UT). The clones were subsequently maintained by weekly passage in the presence of antigen plus rat Con A supernatant.

Cellular Proliferation Assay. The proliferative response was estimated by the incorporation of [^{3}H]TdR, as described (12).

Radiolabeled IL-2 Binding Assay. Radiolabeled IL-2 binding to intact cells was performed essentially as previously described (9). Cells were first separated from debris by centrifugation over a Metrizoate/Ficoll gradient, followed by incubation at 37°C for two 1-h periods in RPMI 1640 with extensive washes between incubations to facilitate removal or degradation of bound IL-2. Serial dilutions of ^{125}I-labeled human rIL-2 (New England Nuclear, Boston, MA) were incubated with 2–8 × 10^5 cells in binding buffer (RPMI 1640, 25 nM Hepes, 10 mg/ml BSA, 0.1% NaN_3) for 15 min in a total volume of 100 μl. After 15 min, 1 ml of cold binding buffer was added and the cells were centrifuged at 9,000 *g* for 20 s in a microfuge. The supernatant was then removed and counted. The cells were washed with an additional 1 ml, then resuspended in 100 μl of binding buffer, underlayed with 200 μl of a 2:5 mixture of Metrizoate/Ficoll to binding buffer, and centrifuged for 90 s at 9,000 *g*. The tips of the tubes containing the cell pellet were cut off and counted in a gamma counter. Nonsaturable binding was determined by inclusion of a 150-fold molar excess of unlabeled human rIL-2 (kindly provided by Drs. Bruce Devins and David Webb, Roche Institute of Molecular Biology, Nutley, NJ).

Northern Blots. Total RNA was prepared according to the method of Chirgwin et al. (13). For Northern blot hybridization, 10–15 μg of RNA from each sample was heated at 65°C in 50% formamide/6.6% formaldehyde in 4-morpholine-propane sulfonic acid (MOPS) buffer and subjected to electrophoresis in a 0.8% agarose gel containing 6.6% formaldehyde. After electrophoresis, the RNA was transferred to Magnagraph nylon hybridization paper (MSI Micron Separations Inc., Westboro, MA). Hybridization was carried out at 42°C for 18 h with probes containing radioactivity of 2–5 × 10^7 cpm. Blots were washed three times in 2× SSC, 0.1% SDS at ambient temperature for 15 min each wash and two times in 0.2× SSC,

0.1% SDS at 56°C for 15 min each wash. Blots were exposed to Kodak XAR film at −70°C for 2–3 d.

DNA Probes. All probes utilized in this study were purified inserts isolated after restriction enzyme digestion, electrophoresis in 1% agarose, and extraction by electroelution. The v-fms probe was a 1.5-kb fragment of v-fms cDNA provided by Dr. Jeff Milbrandt (Washington University, St. Louis, MO). The p53 probe was a 745-bp fragment excised from a cDNA clone of mouse p53 provided by Dr. A. Bernstein (Mount Sinai Hospital Research Institute, Toronto, Ontario, Canada). C-myb was a 500-bp fragment from a mouse cDNA clone provided by Dr. G. Shen-Ong (National Cancer Institute, Bethesda, MD). C-myc was a 1.8-kb fragment of a cDNA clone to mouse c-myc provided by Dr. M. Cole (Princeton University, Princeton, NJ). The probe for lck was a 1,750-bp fragment of a cDNA clone to mouse lck provided by Dr. R. Perlmutter (University of Washington, Seattle, WA). c-abl was a 4.4-bp fragment of a cDNA clone to mouse c-abl provided by Dr. A. Bernards and Dr. D. Baltimore (Whitehead Institute for Biomedical Research, Cambridge, MA). The MHC (K^d) gene probe was a 852-bp fragment of a cDNA clone to the mouse K^d gene provided by P. Kourilsky (Pasteur Institute, Paris, France).

Antisense Oligodeoxynucleotide Inhibition. Antisense oligodeoxynucleotides complementary to the 5′ end of the mRNA coding for mouse c-myb and c-myc were synthesized on a multiple column DNA synthesizer. The choice of sites for these complementary sequences was based on published observations (14, 15). Cells (6 d after antigenic stimulation) were pre-incubated at 4×10^5 cells/well for 2 h in AIM-V, serum-free medium (Gibco Laboratories, Grand Island, NY) alone or with c-myb antisense oligodeoxynucleotide (5′-GGGTCTCCGGGCCAT-3′), c-myc antisense oligodeoxynucleotide (5′-CACGTTGAGGGGCAT-3′), or a scrambled c-myb nonsense oligodeoxynucleotide (5′-CTGCAGTGGCCTGCG-3′) at 30 μM. After 2 h the cells were subcultured into 96-well plates at 4×10^4 cells/well with 50 pM human rIL-2 for 24 h, then pulsed for 6 h with 1 μCi [^{3}H]TdR/well. The cultures were harvested onto glass fiber filters and counted by liquid scintillation. In analyses of the dose dependence of c-myb antisense oligodeoxynucleotide inhibition, significant specific inhibition was observed at oligodeoxynucleotide concentrations as low as 2.4 μM and optimum inhibition was demonstrable at concentrations ranging from 10 to 50 μM.

Anti-TCR Stimulation. Cells were stimulated with anti-TCR mAb as previously described (16). Briefly, tissue culture plates (6-well plates for subsequent Northern analysis or 96-well plates for proliferation assay) were treated with 8 μg/ml rat anti-mouse Ig (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) at 37°C overnight. The plates were then washed with media to remove unbound antibodies and incubated with a 1:10 dilution of F23.1 hybridoma supernatant for 1 h at 37°C. After washing off unbound F23.1 antibody, cloned T lymphocytes were added and incubated at 37°C.

After incubating the cells in F23-coated plates, cells for Northern analysis were resuspended, washed twice with media, and their total RNA was extracted (13). Cells in F23-coated 96-well plates for analysis of proliferation were pulsed for 6 h with 1 μCi/well [^{3}H]TdR.

Results

Transcriptional Expression of the Proto-oncogenes p53, lck, c-abl, c-fms, c-myc, and c-myb in T Lymphocyte Clones with Time after Antigenic Stimulation. We previously reported (9) that antigen-driven cytolytic T lymphocyte clones proliferate vigorously in response to IL-2 early after antigenic stimulation (days 1–3). Late (days 5–8) after stimulation, the clones' responsiveness to the IL-2-proliferative stimulus wanes, and this unresponsiveness is associated with a 10–50-fold drop in the expression of the IL-2-R p55 protein (3). Despite this, relatively high levels of high affinity IL-2-R remain on these quiescent cells (9). Table I shows the IL-2-R sites per cell and the proliferative response of three influenza-specific, class I MHC-restricted cytolytic T lymphocyte clones at day 3 and days 10–14 after antigenic stimulation. Early after antigenic

TABLE I

Proliferative Response of Three Influenza-specific, Class I MHC-restricted Cytolytic T Lymphocyte Clones

Clone	Day after antigenic stimulation	High affinity IL-2-R*	[^{3}H]TdR incorporation‡	
			Media	500 pM IL-2
		sites/cell	*cpm*	
14-13	3	6,300	47 ± 20	185,586 ± 14,372
	10	4,350	70 ± 20	4,633 ± 206
11-1	3	5,952	447 ± 49	191,193 ± 12,146
	10	2,550	400 ± 26	3,064 ± 197
14-7	3	5,100	103 ± 14	215,588 ± 10,697
	14	2,326	94 ± 51	1,054 ± 89

* IL-2 binding analysis was conducted as discussed previously (9).

‡ Cells (at early and late times after antigenic stimulation) were incubated in 96-well plates at 10^4 cells/well for 48 h in media alone or in 500 pM human rIL-2. Values are the mean cpm ± SEM of quadruplicate cultures after a 4-h pulse with 1 μCi [^{3}H]TdR/well.

stimulation the cells vigorously proliferate in response to IL-2. However, by day 10 after stimulation, little proliferation is evident, even though these cells have 42–69% of the level of high affinity IL-2-R as seen early after antigenic stimulation. Although unable to transmit a proliferative signal, the high affinity IL-2-Rs on these quiescent cells are functional by several criteria (9; and see below).

Since the high affinity IL-2-Rs were still capable of signal transduction late after antigenic stimulation, we reasoned that an essential regulatory element necessary for movement of the T cells into S phase may not be expressed (or activated) as a result of the IL-2/IL-2-R interaction in these quiescent cells. Since a number of cellular proto-oncogenes have been implicated as important regulators of lymphocyte growth (17), we considered the possibility that the unresponsiveness of the T cells to IL-2 late after antigenic stimulation might be linked to a block of expression of one or more of these cellular proto-oncogenes. To investigate this hypothesis we screened a panel of clones for transcriptional expression of a number of proto-oncogenes at day 3 after antigenic stimulation, when they proliferate vigorously to IL-2, and day 10–14 after stimulation, when they are refractory to the proliferative stimulus of IL-2. The proto-oncogenes selected (c-*abl*, c-*fms*, *lck*, p*53*, c-*myc*, and c-*myb*) have been previously implicated in the regulation of lymphocyte growth and activation (18–25). Total RNA was extracted from T lymphocyte clones at the early and late time points and examined by Northern analysis. As shown in Fig. 1, transcription of the proto-oncogenes c-*abl*, c-*fms*, *lck*, and p*53* in clones 14-13 and 14-7 was comparable at early and late times. The continued high level expression of these genes late after activation suggests that although these genes may be necessary for cellular proliferation, they may not be playing a critical regulatory role in the IL-2/IL-2-R signal in quiescent clones leading to progression through the cell cycle. C-*myc* transcripts are also readily detectable at day 3 in both 14-13 and 14-7. At the late time point (day 10–12), c-*myc* expression is detectable in both clones but at reduced levels, as is evident in clone 14-7 at day 12 after stimulation. On the other hand, the c-*myb* proto-oncogene was expressed at day 3 after activation but was not detectable late

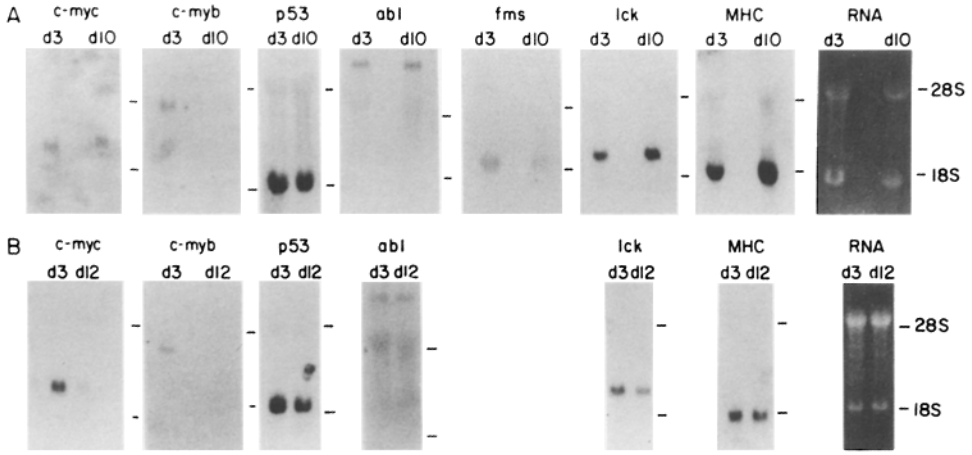

FIGURE 1. Proto-oncogene transcriptional expression at early and late times after antigenic stimulation of class I MHC-restricted cytolytic T lymphocyte clones 14-13 (*A*) and 14-7 (*B*). Total RNA was extracted from cells early and late after antigenic stimulation, and the expression of various proto-oncogenes was assessed by Northern analysis.

after activation. The low level of c-*myc* and the undetectable level of c-*myb* transcriptional expression in both clones late after antigenic stimulation raised the possibility that expression of one or both of these nuclear proto-oncogenes might be causally related to the IL-2-driven proliferative stimulus.

The Expression of the Proto-oncogenes c-myc and c-*myb*, in Response to IL-2 in Early and Late Activated T Lymphocytes. One explanation for the low expression of c-*myc* and c-*myb* transcripts late after activation was that the exogenous IL-2 in the late culture had been exhausted. We tested this by subculturing quiescent (day 10–14) T lymphocyte clones in a supraoptimal concentration of rIL-2 (500 pM) and then examined expression of the c-*myc* and c-*myb* genes at various times after IL-2 exposure. To verify the impact of IL-2 on c-*myc* and c-*myb* expression in activated clones, we exposed cells taken day 3 after antigenic stimulation to an identical regimen of subculture in 500 pM rIL-2. As shown in Fig. 2 for three T lymphocyte clones, expression of the c-*myc* gene markedly unregulated in activated (day 3) clones after exposure to IL-2 with maximal expression demonstrable at ~4 h. Surprisingly, in quiescent clones (day 10–14), c-*myc* was inducible to virtually the same level seen in activated cells in spite of the absence of significant IL-2-dependent proliferation in these quiescent cells (see Table I). In contrast, while expression of the c-*myb* gene was inducible by IL-2 in the activated clonal population (with maximum expression after 8 h of exposure to IL-2), no c-*myb* gene expression was observed in the quiescent clones even after exposure to supraoptimal concentrations of rIL-2. This result was consistent and reproducible even if the amount of total cellular RNA probed was increased by up to fivefold and Northern blots were exposed for prolonged periods (up to 7–10 d).

These observations suggest that the level of c-*myc* gene expression is most likely not the limiting factor in regulating entry of these quiescent T lymphocytes into

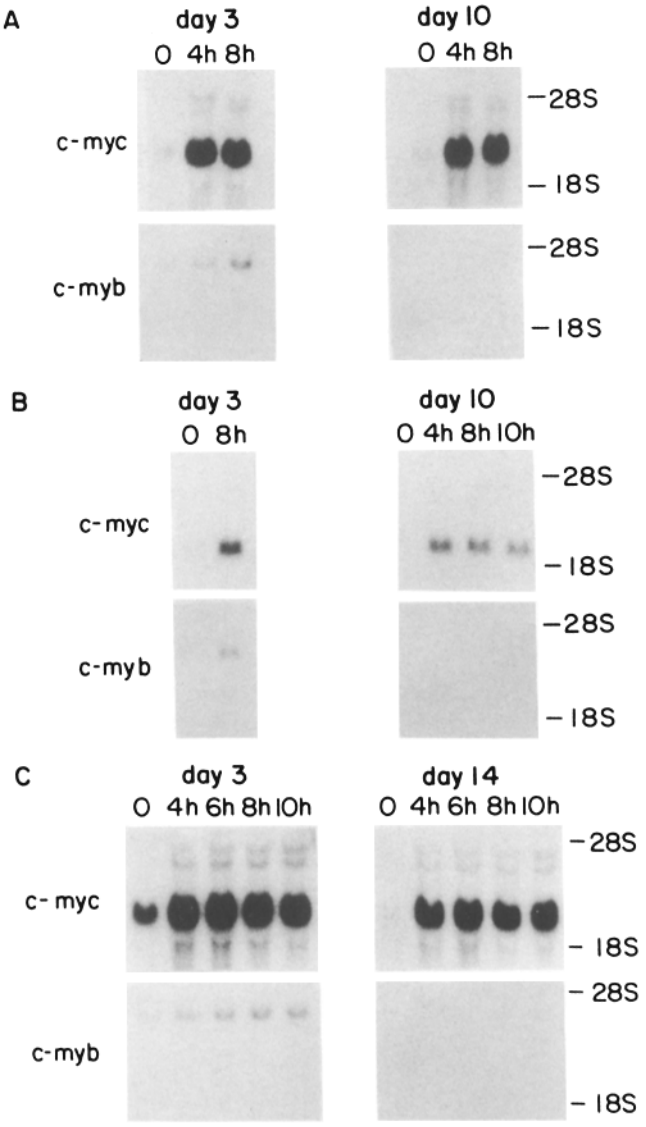

FIGURE 2. Expression of c-*myc* and c-*myb* at early (day 3) and late (day 10–12) times after antigenic stimulation of T lymphocyte clones 14-13 (*A*), 11-1 (*B*), and 14-7 (*C*). At the indicated day after antigenic stimulation, the cells were incubated for the indicated amount of time (in hours) in 500 pM human rIL-2, after which the RNA was extracted and subjected to Northern analysis.

S phase after exposure to IL-2. The marked difference in the expression of c-*myb* in response to IL-2 between early and late activated cells, however, suggested that c-*myb* expression might be critical for cell cycle progression in response to IL-2.

Correlation of IL-2/IL-2-R–dependent Expression of c-myc and c-myb mRNA with IL-2-dependent Proliferation. If c-*myb* plays a central role in regulating the responsiveness of T cells to the IL-2-proliferative stimulus, then the kinetics of c-*myb* expression in the clones should directly parallel the time course of responsiveness of the cells to IL-2. To examine the relationship between the IL-2-dependent proliferative capacity of the clones and the IL-2-dependent expression of the c-*myb* gene, the expression of c-*myb* (along with the c-*myc* gene) was examined at various times after anti-

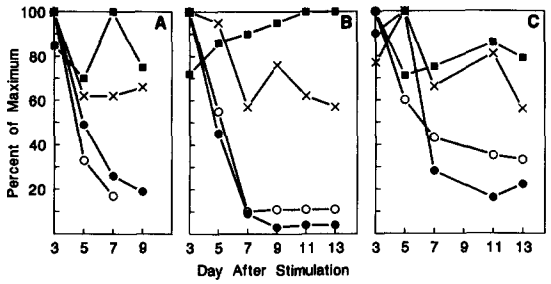

FIGURE 3. Kinetics of IL-2-dependent expression of the proto-oncogenes c-*myb* and c-*myc* and IL-2-dependent proliferation with time after antigenic stimulation of T lymphocyte clones 11-1 (*A*), 14-13 (*B*), and 14-7 (*C*). The T lymphocyte clones were subcultured in 50 pM human rIL-2 at day 2 after antigenic stimulation and every 2 d thereafter. 24 h after subculturing in fresh IL-2, total RNA was extracted and examined for c-*myc* (×) and c-*myb* (○) expression by Northern blotting. The bands were quantitated by densitometry. Simultaneously, the level of IL-2-dependent proliferation was measured by standard proliferation assay (●). The expression of the *MHC class I* gene K^d was included as a control (■).

genic stimulation under conditions of maximum IL-2 availability. To this end the clones were freshly subcultured in saturating concentrations of IL-2 at 2-d intervals for a period of 9–13 d starting at day 2 after antigenic stimulation. The expression of c-*myc* and c-*myb* mRNA and cellular proliferation were then assessed 24 h after subculture. As shown in Fig. 3, the level of c-*myc* over this time period was variable but was never <60–70% of the maximum (day 3) level. IL-2-dependent c-*myb* expression, on the other hand, decayed dramatically with time, directly parallel with the fall in IL-2-dependent cellular proliferation of the clones.

Cell Cycle Progression Is Blocked by an Antisense Oligodeoxynucleotide to c-myb mRNA. The close correlation between c-*myb* expression and IL-2-dependent cellular proliferation supports the view that this gene is critical for the IL-2-dependent cell cycle progression in T lymphocytes. These data are also consistent with an alternate interpretation, i.e., that c-*myb* gene expression is merely a consequence of cellular proliferation or the state of activation of the cell but is not directly required for cell cycle progression in response to IL-2. To directly assess the need for c-myb in movement of the cells into S phase, we examined the effect of an antisense oligodeoxynucleotide complementary to the 5′ region of c-*myb* mRNA on the proliferative response of T lymphocyte clones to IL-2.

As shown in Table II, for clone 14-13, the c-myb antisense oligodeoxynucleotide

TABLE II

Inhibition of Proliferation by Oligonucleotides Complementary to the 5′ Region of mRNA for c-myb and c-myc

Exp.	PBS	Antisense c-myb	Antisense c-myc	c-myb nonsense
1	69,858 ± 4,364*	15,974 ± 1,720 (77.1%)‡	23,534 ± 679 (65.1%)	67,437 ± 1,465 (3.5%)
2	98,009 ± 5,046	18,457 ± 588 (81.2%)	27,288 ± 2,380 (64.1%)	76,001 ± 3,063 (22.5%)

Clone 14-13 6 d after antigenic stimulation was treated as described in Materials and Methods.

* Values are the mean cpm ± SEM from triplicate cultures after a 6-h pulse with 1 μCi [^{3}H]TdR/well.

‡ Numbers in parentheses are percent inhibition based on the amount of proliferation seen with pretreatment with PBS alone.

at a concentration of 30 μM inhibited IL-2-dependent [^{3}H]TdR incorporation by up to 77%. An oligodeoxynucleotide containing the 15 c-myb antisense bases, but arranged in a random order (nonsense oligodeoxynucleotide), gave no significant inhibition of proliferation. In dose-response studies (not shown), antisense c-myb concentrations as low as 2.5 μM inhibited [^{3}H]TdR incorporation by up to 40% with no inhibition by the nonsense oligodeoxynucleotide at this concentration. Maximum inhibition was observed at antisense oligodeoxynucleotide concentrations ranging from 10 to 50 μM. Antisense oligodeoxynucleotides to the 5′ region of c-*myc* mRNA also inhibited IL-2-dependent proliferation (up to 65%), indicating that c-*myc* expression is also necessary for cell cycle progression in these cells. This is in agreement with results reported for human T lymphocytes (14, 15). Antisense oligodeoxynucleotides complementary to other constitutive cellular genes, e.g., antisense β_2-microglobulin, failed to inhibit proliferation (not shown). Our observations support the view that c-*myb* gene expression is not merely coincidental with cellular proliferation but is a necessary element for proliferation in these T lymphocyte clones.

Induction of c-myb Via the TCR. The above data argue for a direct link between c-*myb* gene expression as a result of the IL-2/IL-2-R interaction and movement of T cells into S phase. However, the expression of c-*myb* became progressively refractory to induction via the IL-2-R, so that by day 8–10 after antigenic stimulation, little or no c-*myb* expression was demonstrable in response to IL-2. Since these clones can be activated and rendered sensitive to IL-2 by antigenic stimulation (10), engagement of the TCR would appear to be essential for IL-2-dependent c-*myb* gene expression. However, engagement of the TCR alone has been reported to be sufficient for the induction of certain nuclear proto-oncogenes, e.g., c-*myc* (24). It was therefore possible that antigen receptor engagement could as well lead to c-*myb* induction in quiescent T cells. To test this possibility we directly stimulated the TCR on quiescent clones using the anti-Vβ8-specific mAb F23.1 immobilized on tissue culture plates (16). As Table III shows, stimulation of quiescent cells with antibody in the presence of exogenous IL-2 induces a vigorous proliferative response. Fig. 4 shows an analysis of the kinetics of c-*myb* and c-*myc* induction after exposure of clone 14-13 to the immobilized mAb F23.1 alone. As previously reported (24), the proto-oncogene c-*myc* is efficiently induced within 4 h of engagement of the TCR. Induction of c-*myc* is not dependent on IL-2-R engagement and is demonstrable in the absence of exog-

TABLE III

Induction of Proliferation by Anti-TCR mAb F23.1

Day after stimulation	[^{3}H]TdR incorporation*		
	Media	Secondary antibody	Secondary antibody plus F23
3	483 ± 30	277 ± 35	40,697 ± 400
4	355 ± 22	206 ± 20	18,810 ± 550

* 96-well plates were left untreated, treated with secondary antibody alone, or with secondary antibody and mAb F23.1 as in Materials and Methods. After washing off unbound F23.1, 10^4 cells/well were cultured in the presence of 10% supernatant from Con A-stimulated rat splenocytes as a source of IL-2 and pulsed for 6 h with 1 μCi [^{3}H]TdR. Shown is the peak of proliferation over a 5-d period.

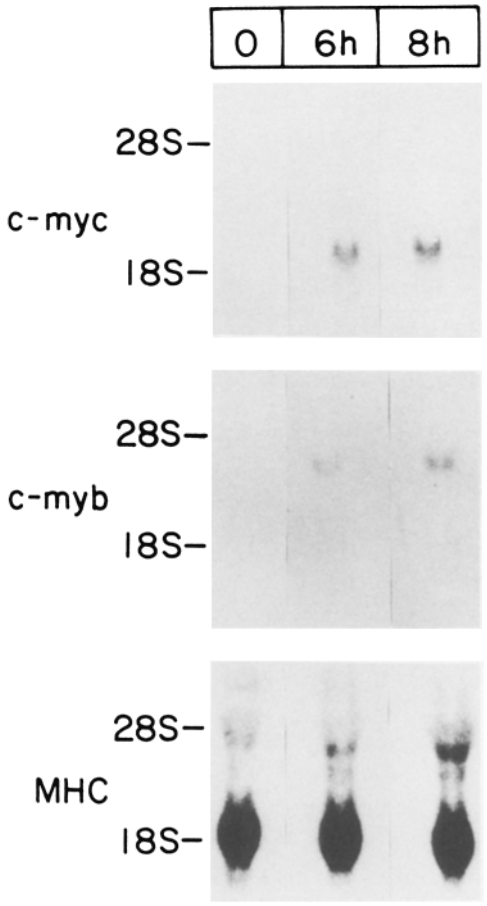

FIGURE 4. Kinetics of c-*myc* and c-*myb* expression after stimulation of clone 14-13 with anti-TCR mAb F23.1. Quiescent cells (day 10 after stimulation) were stimulated with immobilized F23.1 antibody and incubated in culture medium without added IL-2 for 6–8 h at 37°C. At the indicated time after stimulation with F23.1, total RNA was extracted and Northern analysis performed. Expression of the *MHC* K^d gene is included as a control.

enous IL-2. Surprisingly, c-*myb* was also induced by TCR engagement, even in the absence of added IL-2. Northern analysis with a probe to mouse IL-2 demonstrated no detectable IL-2 transcript in the class I CTL clone over this time period (not shown). It thus seems unlikely that the induction of c-*myb* was due to endogenously produced IL-2 derived from the clone.

In conjunction with this analysis we also determined whether de novo protein synthesis was a prerequisite for c-*myb* induction. To this end, clone 14-13 was stimulated with immobilized F23.1 antibody in the presence of the protein synthesis inhibitor cycloheximide. As shown in Fig. 5, c-*myb* was efficiently induced by TCR engagement alone under conditions of 99% inhibition of protein synthesis. The induction of c-*myb* in the presence of the protein synthesis inhibitor further supports the hypothesis that the induction of c-*myb* does not require de novo synthesis of regulatory molecules, e.g., endogenous lymphokines, and implies that the induction signal is transduced via a preformed regulatory molecule.

2-Aminopurine (2-AP)[1] *Inhibits c-myb Induction Via the IL-2-R Pathway but not the TCR*

[1] *Abbreviation used in this paper:* 2-AP, 2-aminopurine.

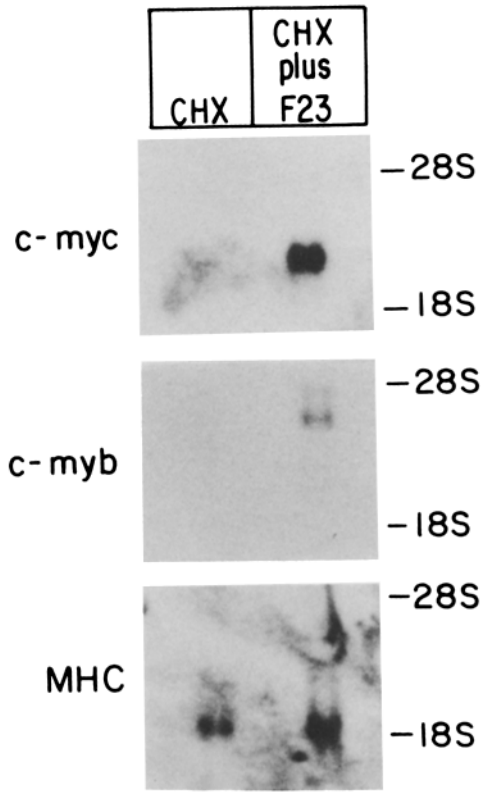

FIGURE 5. Induction of the proto-oncogenes c-*myc* and c-*myb* in the presence of the protein synthesis inhibitor cycloheximide. Clone 14-13 (day 10 after stimulation) was pretreated with cycloheximide (CHX, 40 μg/ml) for 30 min and then cultured in IL-2-free medium in control plates or F23.1 antibody-coated plates. After the 7-h incubation, total RNA was extracted and c-*myc* and c-*myb* RNA levels were determined by Northern analysis.

Pathway. The above observations suggest that c-*myb* can be directly induced via engagement of the TCR and, therefore, that an IL-2-independent pathway exists for the induction of c-*myb*. It was of interest to determine whether the induction of this gene by the TCR and the IL-2-R occurs by a similar mechanism, e.g., a common intermediate.

The antimetabolite 2-AP has been shown to inhibit c-*myc* expression in fibroblast cells undergoing serum-stimulated proliferation (26), presumably due to its ability to inhibit selected cellular protein kinases. In view of the effect of 2-AP on nuclear proto-oncogene expression in serum-stimulated cellular proliferation, it was of interest to determine whether the drug influences the induction of the c-*myc* and c-*myb* proto-oncogenes after engagement of the IL-2-R by IL-2 in activated T cells (day 3 after stimulation). As shown in Fig. 6, 2-AP markedly inhibited IL-2-driven expression of both c-*myc* and c-*myb* in the activated T lymphocyte clone, indicating that the IL-2/IL-2-R–mediated pathway of c-*myc* and c-*myb* induction was sensitive to this inhibition.

A different result emerged when we assessed the effect of 2-AP on c-*myc* and c-*myb* induction mediated by engagement of the TCR. As shown in Fig. 7, 2-AP inhibits expression of c-*myc* induced by engagement of the TCR, while in marked contrast, c-*myb* expression is enhanced. It appears that 2-AP has a differential effect on c-*myb* and c-myc induction after TCR engagement but inhibits the IL-2-induced expres-

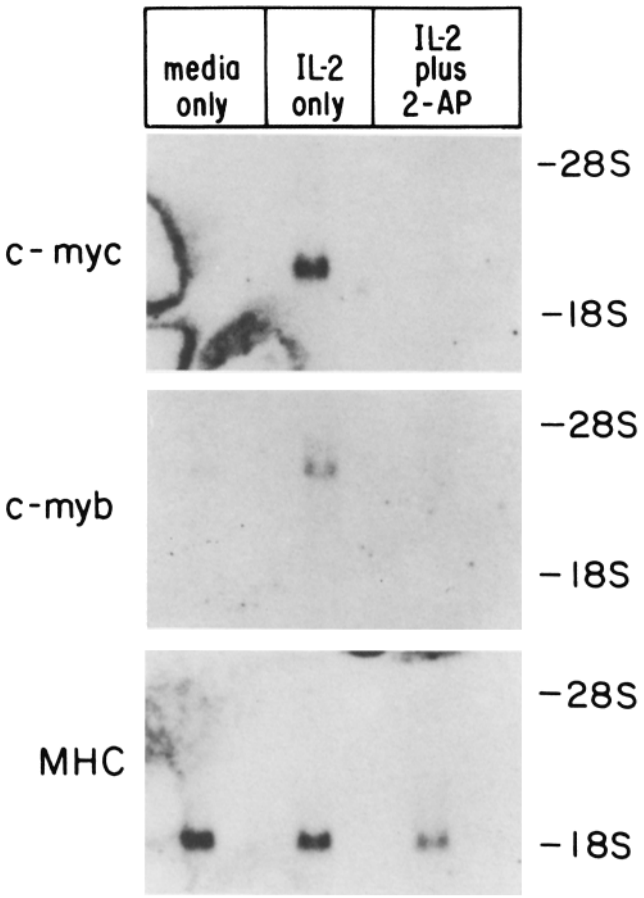

FIGURE 6. The effect of 2-AP on the IL-2-dependent induction of c-*myb* and c-*myc* in activated T lymphocytes. Clone 14-13 (day 3 after antigenic stimulation) was incubated for 7 h in medium supplemented with 10 mM 2-AP alone or 2-AP in combination with 500 pM human rIL-2. Total RNA was then extracted and examined by Northern analysis for the expression of c-*myc* and c-*myb* mRNA.

sion of both these proto-oncogenes. Importantly, 2-AP appeared to have no effect on the expression of other genes, e.g., *MHC class I* (see Fig. 6) constitutively expressed in these cells.

Discussion

Most previous studies on the role of c-*myb* and c-*myc* expression in T lymphocytes have centered on the use of either lectin-stimulated heterogeneous populations of cells (24, 25) or on cell lines or clones that are IL-2 dependent and antigen independent (27). In our studies, we examine the regulation of MHC-restricted, antigen-specific murine T lymphocyte clones. Utilization of these cells enables us to directly assess the mechanisms underlying the control of proliferation in cells that require routine restimulation with antigen (every 7–14 d) for their propagation in culture.

The aim of this investigation was to determine whether responsiveness of T lymphocytes to the proliferative stimulus mediated by IL-2 is linked to the expression of particular proto-oncogene(s). We found that in activated T lymphocyte clones capable of proliferating in response to exogenous IL-2, the c-*myb* proto-oncogene

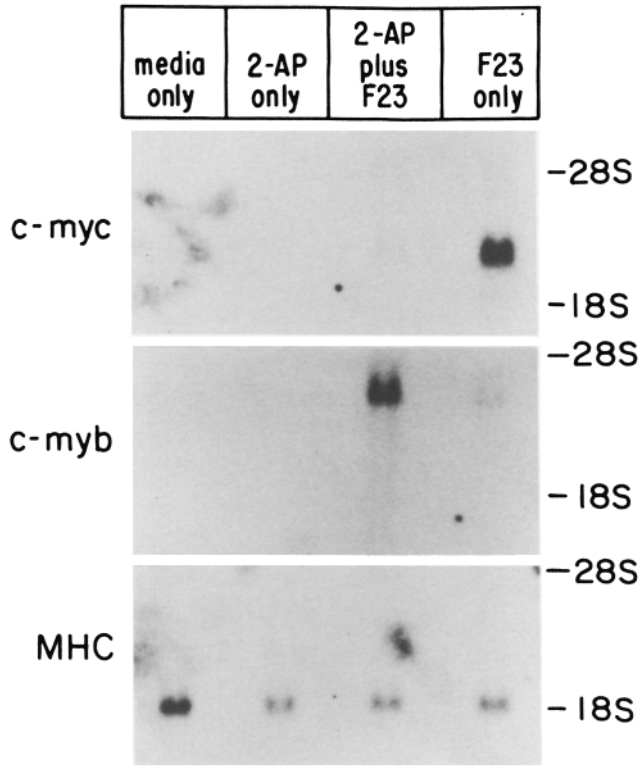

FIGURE 7. The effect of 2-AP on TCR-mediated induction of c-*myb* and c-*myc*. Quiescent (day 11) clone 14-13 cells were stimulated with F23.1 antibody in the presence of 10 mM 2-AP or were incubated in 2-AP supplemented medium alone as described in Table III. Total RNA was extracted and examined by Northern analysis for the expression of c-*myc* and c-*myb* mRNA.

was readily induced by the IL-2/IL-2-R interaction. We also found that quiescent T lymphocyte clones that did not respond to the proliferative stimulus of IL-2 failed to express detectable levels of c-*myb* mRNA after exposure to IL-2. These data implicated c-*myb* as a possible regulator of IL-2-dependent T lymphocyte proliferation. A role for c-*myb* was further supported by the finding of a close temporal correspondence between the extent of DNA synthesis in response to IL-2 and the level of c-*myb* RNA expression in T lymphocytes transiting from an activated phase to a quiescent phase as a function of time after antigenic stimulation. Finally, the importance of c-myb in regulating movement of T lymphocytes into the S phase of the cell cycle was directly demonstrated by specific inhibition of IL-2-driven DNA synthesis by treatment of cells with an antisense c-myb oligodeoxynucleotide. Overall, these data provide strong support for the view that c-*myb* expression is necessary for IL-2-dependent T lymphocyte proliferation, and that the lack of IL-2-induced expression of this proto-oncogene in quiescent T lymphocytes accounts in part for the unresponsiveness of quiescent T lymphocytes to the IL-2-proliferative signal.

That c-*myb* expression is necessary but not sufficient for IL-2-driven T lymphocyte proliferation is evident from the effect of the antisense c-myc oligodeoxynucleotide that also markedly inhibited proliferation. In contrast to c-*myb*, the expression of the c-*myc* proto-oncogene is inducible by IL-2 even in quiescent cells. While its expression is obviously not sufficient for IL-2-dependent proliferation, c-*myc*, how-

ever, appears necessary for movement into the S phase of the cell cycle. A role for c-myc in lymphocyte activation/proliferation has been demonstrated in studies with human (14, 15, 24) and murine (27) T lymphocytes. Our data showing inhibition of lymphocyte proliferation by antisense c-myc oligodeoxynucleotide are consistent with these data.

In this report we show that c-*myb* expression is inducible either by engagement of the T lymphocyte antigen receptor or by engagement of the IL-2-R. Upregulation of c-*myb* expression in T lymphocytes as a result of IL-2-R engagement has been previously reported (25) and a direct dependence of c-*myb* expression on the IL-2/IL-2-R interaction has been suggested (25, 28). Our results are consistent with this notion in that engagement of the IL-2-R on activated T lymphocytes causes a much greater increase in c-*myb* expression than results from TCR engagement (A. Churilla, unpublished observations). C-*myb* expression is also stimulated via the TCR and its induction does not require de novo protein synthesis in the T cell. Thus, the TCR-dependent induction of c-*myb* can not be accounted for by generation of a cytokine, e.g., IL-2, in the T cell, which could then stimulate via the IL-2-R. Also, c-*myb* induction by the IL-2-R pathway is sensitive to 2-AP while the drug actually serves to increase the level of c-*myb* RNA induced by TCR engagement. These results suggest that the mechanisms of c-*myb* induction after TCR and IL-2-R engagement most likely are different.

In view of the capacity of signals from either the TCR or the IL-2-R to induce c-*myb*, it is likely that under usual conditions of T cell activation c-*myb* expression is biphasic with an initial burst of c-*myb* transcription as a result of antigen receptor engagement followed by upregulation of c-*myb* as a result of IL-2-R engagement. The IL-2-R-linked c-*myb* induction is most closely associated with cell cycle progression and cellular proliferation. The significance of antigen receptor–induced c-*myb* expression is less clear. It is noteworthy, however, that quiescent $CD8^+$ T cell clones triggered via the antigen receptor alone appear to be able to synthesize DNA but not progress completely through the cell cycle and undergo proliferation (T. J. Braciale, unpublished observations). C-*myb* itself may, therefore, have both competence and progression functions depending on the state of activation of the cells.

Recent evidence (29, 30) indicates that the IL-2-R can transduce a proliferative signal by a pathway that does not require protein kinase C, whereas TCR-mediated signaling events may be protein kinase C dependent. These findings, like those reported herein, reinforce the notion that intracellular signaling via these two receptor systems are by different mechanisms.

Our finding of inhibition of IL-2-induced c-*myb* expression by the protein kinase inhibitor 2-AP appears to be at variance with the evidence for IL-2 signaling by a protein kinase C–independent pathway. It should be noted, however, that the inhibitory effect of 2-AP appears to be specific for certain classes of protein kinases, e.g., heme-regulated protein kinases (26), and that no direct effect of the 2-AP on protein kinase C has been documented. Since the mechanism of action of 2-AP is not precisely known, the drug may affect multiple sites along the IL-2-signaling pathway. Our observations at least raise the possibility that a protein kinase–mediated phosphorylation event is critical for IL-2-dependent c-*myb* induction and subsequent proliferation.

A number of different mechanisms of regulation of proto-oncogene expression

have been proposed (31–33). Perhaps the simplest hypothesis to account for our findings is that a preformed regulatory factor present in quiescent T lymphocytes is modified (e.g., phosphorylated or dephosphorylated) as a result of TCR engagement. This "competence" (modified regulatory) factor can then promote expression of c-*myb* directly at a low level and would be susceptible to further alteration promulgated as a result of signaling through the IL-2-R. This second modification mediated through the IL-2-R pathway would then allow this further modified or "progression" factor to induce c-*myb* expression with a higher efficiency. Most importantly, while the unmodified form of this regulator would be present in the quiescent cells (and possibly in activated cells), the two modified forms of this regulator must be limiting, i.e., transiently present in activated cells and absent in quiescent cells, in order to account for the time-dependent decay in sensitivity of the T cells to the IL-2-proliferative stimulus (Fig. 3). Models involving de novo expression of IL-2-dependent c-*myb* regulators after TCR engagement likewise can account for these results. These models are highly speculative at present but suggest avenues for further studies on the regulation of T lymphocyte growth.

T lymphocyte activation/proliferation is regulated at several levels. The availability of antigen in a form capable of engaging the TCR represents the primary or most basic form of regulation. With the evolution of knowledge concerning lymphocyte-specific growth factors, it has become clear that the availability of IL-2 and related lymphocyte-specific growth factors as well as the cell surface expression of the relevant hormone receptors represents another layer of regulation of lymphocyte growth. In this report we have obtained evidence for a third, intracellular level of regulation where expression of genes critical for lymphocyte growth, e.g., c-*myb*, is controlled not only at the level of growth factor receptor engagement but also at the level of intracellular modulators of gene expression. Our finding provides a framework for further analysis of the molecular basis of lymphocyte quiescence and also may offer new avenues of investigation for understanding the pathogenesis of disorders such as angioimmunoblastic lymphadenopathy (34) and the autoimmune diseases associated with the homozygous lpr/lpr phenotype in mice where aberrant proto-oncogene expression is linked to abnormal lymphocyte proliferation (35, 36).

Summary

We previously reported that with time, after antigenic stimulation of antigen-regulated murine T lymphocyte clones, total IL-2-R expression decayed 10–50-fold, commensurate with a decline in the ability of the cells to proliferate to IL-2. However, late after antigenic stimulation, when the cells were refractory to the IL-2-proliferative stimulus, high levels of high affinity IL-2-R remained.

In this report we further explore the basis of unresponsiveness to IL-2 in the quiescent clones. We show that the proto-oncogene c-*myc* is induced in the late cell population by IL-2 to comparable levels observed early after antigen stimulation. IL-2-dependent c-*myb* induction, however, is seen only early after activation but not in the late-activated population. Analysis of the IL-2-dependent expression of c-*myb* mRNA with time after antigenic stimulation showed that steadystate c-*myb* expression declines dramatically with kinetics closely paralleling a decay in IL-2-dependent proliferative ability. In contrast, steadystate c-*myc* expression remains high throughout this period. Expression of c-myb is critical for proliferation of these cells since antisense oligodeoxy-

nucleotide to c-myb can inhibit their IL-2-dependent proliferation. We present evidence for a pathway of c-*myb* induction via the TCR that is independent of the IL-2/IL-2-R interaction. In addition, the inhibition of IL-2-R-induced c-*myb* expression by 2-aminopurine and enhanced induction of c-*myb* via the TCR demonstrate that TCR activation and IL-2-R activation lead to induction of c-*myb* by different mechanisms.

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