Ca\(^{2+}\)/Calmodulin-dependent and -independent Down-regulation of c\(-myb\) mRNA Levels in Erythropoietin-responsive Murine Erythroleukemia Cells

THE ROLE OF CALCINEURIN*

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Down-regulation of c\(-myb\) mRNA levels by [Ca\(^{2+}\)]-increasing agents (A23187, thapsigargin, cyclopiazonic acid) and erythropoietin was comparatively studied in the erythropoietin-responsive murine erythroleukemia cell line, ELM-I-1. The Ca\(^{2+}\)-induced suppression of c\(-myb\) mRNA could be inhibited by the calmodulin antagonists trifluoperazine and calmidazolium, as well as by cyclosporin A, an inhibitor of the Ca\(^{2+}\)/calmodulin-dependent protein phosphatase 2B (calcineurin). KN-62, an inhibitor of Ca\(^{2+}\)/calmodulin-dependent protein kinases, did not antagonize the Ca\(^{2+}\)-mediated decrease in c\(-myb\) mRNA. In cyclosporin A-treated ELM-I-1 cells, a close correlation could be demonstrated between the antagonization of the Ca\(^{2+}\) effect on c\(-myb\) mRNA levels and inhibition of the calcineurin phosphatase activity. On the other hand, FK506, which did not inhibit calcineurin activity in ELM-I-1 cells, failed to prevent the Ca\(^{2+}\)-mediated decrease in c\(-myb\) mRNA. The erythropoietin-induced down-regulation of c\(-myb\) mRNA levels could be demonstrated also in the presence of EGTA and was resistant to calmodulin antagonists and cyclosporin A. In addition, no increase in [Ca\(^{2+}\)] was resistant to calmodulin antagonists and cyclosporin A, while these effects of erythropoietin occur independently.

The modulation of the expression of c\(-myb\) and c\(-myc\) protooncogenes has been implicated in the physiological signal pathways of growth and differentiation of erythroid precursor cells (1). Erythropoietin (Epo),\(^1\) the principal regulator of erythropoiesis, induces a rapid up-regulation of c\(-myc\) and a simultaneous down-regulation of c\(-myb\) mRNA levels (2–5). These protooncogene responses occur via two discrete signaling pathways. The effect of Epo on c\(-myc\) expression is prevented by inhibitors of protein kinase C, while the signal to c\(-myb\) is prevented by okadaic acid, an inhibitor of the serine/threonine-specific protein phosphatases 1 and 2A (5).

The precise role of the c\(-myb\) and c\(-myc\) responses in the effect of Epo on cell growth and differentiation has not yet been clarified. Treatment of Rauscher erythroleukemia cells with an antisense oligodeoxynucleotide to c\(-myb\) results in induction of hemoglobin synthesis without any significant effect on cell proliferation (6). These results suggest that the suppression of c\(-myb\) expression is related to the differentiation-inducing activity of Epo. In fact, a common feature of the action of Epo and dimethyl sulfoxide (Me\(^2\)SO)-like chemical inducers of the differentiation in erythrocyte precursors is the early down-regulation of c\(-myb\) mRNA levels (2–5). Me\(^2\)SO, in contrast to Epo, induces a decrease also in c\(-myc\) expression.

In previous studies we reported that [Ca\(^{2+}\)]-increasing agents, such as Ca\(^{2+}\)-ionophores and inhibitors of the endoplasmic reticulum Ca\(^{2+}\)-pump, induce in Friend erythroleukemia cells an early and transient decrease in c\(-myb\) mRNA levels without similar changes in the c\(-myc\) expression (7, 8). The early decrease in c\(-myb\) mRNA was followed by induction of \(\beta\)-globin mRNA and hemoglobin synthesis. These results support the view that the early down-regulation of c\(-myb\) expression is involved in the induction of the erythroid differentiation pathway and provide further insight into biochemical mechanisms regulating c\(-myb\) expression in erythroid precursor cells. Since Epo has been shown in many studies to induce an increase in [Ca\(^{2+}\)] (9–11), the question arises whether the Epo-induced down-regulation of the c\(-myb\) expression is mediated by a Ca\(^{2+}\) signal.

The aim of the present investigations was to further analyze the down-regulation of c\(-myb\) mRNA levels and induction of hemoglobin synthesis by [Ca\(^{2+}\)]-increasing agents and to compare the Ca\(^{2+}\)-induced changes with the action of Epo in the Epo-responsive murine erythroleukemia cell line ELM-I-1 (12).

MATERIALS AND METHODS

Chemicals—A23187, cyclopiazonic acid, and thapsigargin were purchased from Sigma, trifluoperazine and calmidazolium from Biomol (Plymouth Meeting, PA), okadaic acid from Life Technologies, Inc. and from Sigma, KN-62 and fura-2/AM from Calbiochem; cyclosporin A was obtained from Sandoz (Basel, Switzerland), FK506 from Fujisawa (Tokyo, Japan), and mouse and human recombinant erythropoietin from

\(^1\) The abbreviations used are: Epo, erythropoietin; CaM, calmodulin; CMZ, calmidazolium; CPA, cyclopiazonic acid; CSA, cyclosporin A; CRE, cAMP response element; CREB, cAMP response element-binding protein; kb, kilobase(s); Me\(^2\)SO, dimethyl sulfoxide; NGF, nerve growth factor-induced gene; PP, protein phosphatase.
Boehringer (Mannheim, Federal Republic of Germany). 

\[ \text{[\(\alpha\text{-32P}\)dCTP (3000 Ci/mmole) and [\(\gamma\text{-32P}\)ATP (4500 Ci/mmole)] were obtained from Amesham (Buckinghamshire, United Kingdom) and ICN Pharmaceuticals (Irvine, CA); } \]

\[ \alpha \text{ minimal essential medium without nucleosides, Ham's F-12 medium, horse serum, fetal calf serum, and trypan blue solution were purchased from Life Technologies, Inc., benzidine and bovine hemoglobin from Sigma, analytical grade chemicals from Sigma, Fluka (Buchs, Switzerland), and Merck (Darmstadt, FRG).} \]

Cells and Culture Conditions—Erythropoietin-responsive murine erythroleukemia cells, line ELM-I-1 (12, 13) and Friend erythroleukemia cells, line F4-6 (14) were kindly provided by Prof. W. Oster tag, Heinrich Pette Institute for Experimental Virology and Immunology (Hamburg, FRG). Cells were grown in a medium without nucleosides, supplemented with 2 mM glutamine, 50 units/ml penicillin, and 50 \(\mu\)g/ml streptomycin and with 10% horse serum (ELM-I-1 cells) or with 10% fetal calf serum (F-4-6 cells) at 37 °C in a humidified atmosphere containing 5% CO₂.

For the experiments, exponentially growing cells were plated at 6–8 \(\times\) 10⁵ cells/ml. Approximately 16 h later, the cells were treated with the test substances and at defined time points thereafter, cells were harvested and stored at −80 °C before RNA isolation. For protein isolation, cells were washed in ice-cold phosphate-buffer saline and suspended in phosphate-buffered saline with 6% trichloroacetic acid and stored at −80 °C. To measure hemoglobin production, ELM-I-1 cells were incubated for 3 days, F-4-6 cells for 4 days with the test substances, and hemoglobin content of the cells was determined by the benzidine technique of Luftbg et al. (15) using bovine hemoglobin as standard. Cell viability was examined by trypan blue exclusion. Serum-free incubation of total cellular RNA was isolated and tested using Northern blot analysis.

Northern Blot Analysis—Total cellular RNA was isolated by the acid guanidium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (16). 30-μg samples of RNA were denaturated by glyoxylation, size-separated by electrophoresis through 1% agarose gel, and transferred to a Biodine A membrane (Pall, Portsmouth, UK) by the capillary blotting technique (17) using 20 × standard saline citrate solution (SSC, 1 × SSC contains 0.15 M sodium chloride and 0.015 M sodium citrate). RNA was immobilized by baking the membrane at 80 °C for 1.5 h. DNA probes for hybridization were labeled with \([\alpha\text{-32P}]\)ATP by the random primer method (18) using the multiprimed DNA labeling system from Amersham. Prehybridization (6–8 h) and hybridization (18–20 h) were carried out at 42 °C in 50% formamide, 50 mM sodium citrate, 5 × SSC, 5 × Denhardt’s solution (0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone), 0.2% SDS, and 250 μg/ml denatured her Harr sperm DNA (Boehringer). Hybridized blots were washed in 2 × SSC and 0.1% SDS at room temperature for 20 min and at 50 °C for 60 min, in 0.5 × SSC and 0.1% SDS at 50 °C for 30 min, and finally in 0.2 × SSC and 0.1% SDS at 55 °C for 30 min. The membranes were exposed to −80 °C on an x-ray film using intensifying screens.

The following hybridization probes were used: human \(\beta\)-actin cDNA, a 1.2-kb probe. It was kindly provided by Prof. T. Braun (Institute of Biochemistry and Biotechnology, University of Braunschweig, Braunschweig, FRG); human c-myb cDNA (19), a 1.2-kb BamH I fragment isolated from the pHM1 construct (20) was kindly donated by Dr. J. Nowock (Heinrich Pette Institute of Experimental Virology and Immunology, Hamburg, FRG); mouse \(\mu\)m-globin DNA (21) was generously donated by Dr. J. Nowock (Heinrich Pette Institute of Experimental Virology and Immunology, Hamburg, FRG). A 1.0-kb BamH I fragment with coding sequences from the structural gene was used. Human c-myc exon 3, a 1.4-kb ClaI-EcoRI fragment, was purchased from Oncor (Gaithersburg, MD).

We carried out the quantitative evaluation of the blots using a Hewlett-Packard Scanjet II CXT flatbed scanner and the software NIH image 1.56 as described previously (8).

Western Blot Analysis—Trichloroacetic acid-precipitated cell material was collected by centrifugation, solubilized in 8 M urea, and diluted with the same volume of denaturation buffer containing 50 mM Tris-phosphate, pH 6.8, 6 M SDS, 2% mercaptoethanol, 20 mM EDTA, and 0.5% sodium dodecyl sulfate (SDS) and incubated at 85 °C for 1 min in a water bath. Protein content of the samples was determined according to Lowry et al. (22). Portions of 60 μg of total cell protein were separated by electrophoresis on a 10% SDS-polyacrylamide gel (23) and transferred to Biodin A membrane (Pall) using the Mini-Protein II system from Bio-Rad. c-Myb protein was detected with a rabbit anti-c-Myb polyclonal antibody (Medac, Hamburg, FRG) and an anti-rabbit IgG peroxidase conjugate (Sigma) as secondary antibody using the ECL Western blotting system of Amersham.

\([\text{[Ca}^{2+}]\text{i}] \text{ Measurements—Fluorescence [\text{Ca}^{2+}] \text{ measurements and cal-}\]

\([\text{ulations were carried out as described previously (8). Briefly, cells (2.5–5 \times 10⁷ cells/ml) were loaded in culture medium with 1.5 μM of fura-2/AM fluorescent calcium indicator at 37 °C for 30 min. Before each measurement, an aliquot (1 ml) of the} \]

\([\text{loaded cell suspension was rapidly centrifuged (10 s at 12,000 × g} \]

\([\text{in an Eppendorf microcentri-}\]

\([\text{fuge, and the pellet was rinsed three times with the standard} \]

\([\text{measuring medium, containing 120 mM NaCl, 5 mM KCl, 0.5 mM MgCl₂, 0.04 mM CaCl₂, 10 mM Heps-Na, pH 7.4, 10 mM NaHCO₃, 5 mM NaHPO₄, and 10 mM glucose and resuspended in 2 ml of measuring medium (2.25–5 × 10⁶ cells/ml). Fluorescence was measured in a Hitachi F-4000}}\]

\([\text{fluorescence spectrophotometer at 37 °C (excitation wavelength, 340 nm; emission wavelength, 500 nm; bandwidth, 5 nm). Cytoplasmic free}}\]

\([\text{calcium concentration was calculated by using the method of Tsien et al. (24).}}\]

Phosphatase Assay—Cells were treated with test substances and calcium in phosphatase activity in hypotonic lysates was measured by the methods of Fruman et al. (25) using the RI peptide substrate (Alexis Corp., Läufelfingen, Switzerland) labeled with \([\gamma\text{-32P}]\)ATP and the catalytic subunit of bovine cardiac CAMP-dependent protein kinase (Sigma) (26). Assays were performed in duplicate, and calcineurin activity was determined as Ca²⁺-dependent phosphatase activity in the presence of 500 μM 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). Protein concentration was determined by the method of Lowry et al. (22).

RESULTS

Effect of Calmodulin Antagonists on the Ca²⁺-Induced Decrease in c-myb mRNA—In accordance with previous results in Friend erythroleukemia cells, line F4-6 (7, 8), [Ca²⁺]-increasing agents (A23187, thapsigargin, cyclopiazonic acid) induced a rapid decrease in c-myb mRNA levels in ELM-I-1 cells, too. In order to study whether calmodulin (CaM) is involved in suppression of c-myb expression by [Ca²⁺]-increasing agents, experiments were performed with the known chemically unrelated CaM antagonists, trifluoperazine and calmidazolium (27). As shown in Fig. 1, 50 μM trifluoperazine and 5 μM calmidazolium antagonized the effect of thapsigargin (2 nM) on the c-myb mRNA levels during a 3-h incubation.

Effect of Inhibitors of Ca²⁺/calmodulin-dependent Protein Kinases and Protein Phosphatase on the Ca²⁺-Induced c-myb mRNA Decrease—The results with the CaM antagonists sug-
For experimental conditions, see the legend to Fig. 1.

...showed that the down-regulation of c-myb mRNA levels by [Ca\(^{2+}\)]-increasing agents is mediated by Ca\(^{2+}\)/CaM-dependent enzymes. In the experiment shown in Fig. 2 we investigated the effect of KN-62, an inhibitor of the Ca\(^{2+}\)/CaM-dependent protein kinase II and other members of the Ca\(^{2+}\)/CaM-dependent kinase family (28–30). KN-62, at concentrations active in cell cultures, 3 and 6 \(\mu\)M, did not inhibit the suppression of c-myb mRNA levels by A23187, 1.5 \(\mu\)M. In further experiments, cyclosporin A and FK506, specific inhibitors of the Ca\(^{2+}\)/CaM-dependent protein phosphatase, PP2B, or calcineurin in complex with immunophilins (31, 32) were investigated. Cyclosporin A (CsA) proved to be a potent antagonist of the Ca\(^{2+}\)/CaM-dependent protein phosphatase activity in serum-free medium and in the presence and absence of A23187, 1.5 \(\mu\)M (Fig. 2). FK506 at concentrations of 200 and 1000 \(\text{nM}\) (Fig. 3) did not inhibit the suppression of c-myb mRNA levels by A23187, 1.5 \(\mu\)M. In the absence of A23187, c-myb mRNA levels were not significantly influenced by CsA (Fig. 3A). In the presence of A23187, CsA inhibited the decrease in c-myb mRNA already at the lowest concentration tested (Fig. 3B). The quantitative evaluation of the blots showed that the Ca\(^{2+}\)-induced decrease in c-myb mRNA was inhibited to 80% at 40 \(\text{nM}\) CsA and completely abolished at 100 and 250 \(\text{nM}\) CsA. On the other hand, FK506 failed to antagonize the Ca\(^{2+}\)-induced down-regulation of c-myb mRNA levels, even at the highest concentrations of 200 and 1000 \(\text{nM}\) (Fig. 3C).

Studies on the Effect of Cyclosporin A and FK506 on the Calcineurin Phosphatase Activity—ELM-I-1 cells were exposed to CsA and FK506 for 1 h, and hypotonic cell lysates were then made and assessed for PP2B activity (25). The results are summarized in Fig. 4. CsA inhibited the calcineurin phosphatase activity in a concentration range of 3–200 \(\text{nM}\) with approximately the same effectiveness as the Ca\(^{2+}\)-induced down-regulation of c-myb mRNA levels (Fig. 3B). FK506 at concentrations up to 2000 \(\text{nM}\) gave no appreciable inhibition correlating with its lack of ability to prevent the Ca\(^{2+}\) effect on the c-myb expression (Fig. 3C). Parallel, we measured calcineurin phosphatase activity in Jurkat T cells under the same experimental conditions and ensured that the FK506 used was active in inhibiting phosphatase activity in this cell line (data not shown). Similarly to the results of previous studies (25, 33, 34), 15–20% of the net phosphatase activity measured was resistant to 200 \(\text{nM}\) CsA in both cell lines and to 200 \(\text{nM}\) FK506 in Jurkat cells.

Effects of Erythropoietin on c-myb and c-myc Expression in ELM-I-1 Cells—In order to compare the Ca\(^{2+}\)-induced down-regulation of c-myb mRNA with the action of Epo in the Epo-sensitive ELM-I-1 cell line (12, 13), we studied the effects of Epo on the c-myb and c-myc expression. Fig. 5A shows the time course of the effect of Epo, 1 unit/ml, on c-myb and c-myc mRNA levels during an incubation period of 24 h. In accordance with studies in other murine erythroleukemia cell lines (2–5), Epo induced a rapid decline in c-myb mRNA levels. A maximal drop (to approximately 20% of control) was reached within 2 h. c-myb mRNA levels remained decreased during the 24-h incubation period at 20–50% of control. The c-myc mRNA showed a slight transient increase at 1 h (135% of control). The Epo-induced suppression of c-myb expression was confirmed in the present study by Western blot analysis of the c-Myc protein during the first 12 h of incubation (Fig. 5B).

The effect of Epo, 1 unit/ml, on c-myc expression was also studied in serum-free medium and in the presence and absence of EGTA, 3 \(\text{mM}\). The results shown in Fig. 6 indicate that the Epo effect on c-myc expression occurred independently of other serum factors or of extracellular Ca\(^{2+}\). In accordance with previous results in F4-6 cells (8), EGTA caused an increase in c-myb mRNA at 3 h. In the same experiment, a strong increase in \(\beta\)-globin mRNA was demonstrated in cultures treated with Epo in serum-free medium for 36 h.
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FIG. 5. Studies on the effects of Epo on c-myb and c-myc expression in ELM-I-1 cells. A, time course of c-myc, c-myb, and \(\beta\)-actin mRNA levels during a 24-h incubation period with Epo, 1 unit/ml. For experimental conditions, see the legend to Fig. 1. B, Western blot analysis of c-myc protein levels during a 12-h incubation period with Epo, 1 unit/ml. Amounts of 60 \(\mu\)g of total cellular protein were separated by electrophoresis on a 10% SDS-polyacrylamide gel, transferred to a nylon filter, and analyzed as described under "Materials and Methods."

FIG. 6. Studies on the effects of Epo in serum- and Ca\textsuperscript{2+}-free medium. Levels of c-myc and \(\beta\)-actin mRNA in ELM-I-1 cells after a 3-h incubation with Epo, 1 unit/ml, in the presence and absence of EGTA. 3 mm, and levels of \(\beta\)-globin and \(\beta\)-actin mRNA after 36-h incubation without EGTA. Cells were preincubated in serum-free medium for 16 h before drug treatments, and incubation was continued in serum-free medium. For experimental conditions, see the legend to Fig. 1.

Studies on the Effect of Calmodulin Antagonists and of Cyclosporin A on the Erythropoietin-induced Decrease in c-myb mRNA—In further experiments, the Ca\textsuperscript{2+} antagonists, trifluoperazine and calmidazolium, as well as the calcineurin inhibitor CsA, were investigated for their ability to prevent the effect of Epo on c-myb mRNA levels in ELM-I-1 cells. The inhibitors were tested at concentrations known to be active in antagonizing the suppression of c-myc mRNA by [Ca\textsuperscript{2+}]-increasing agents without significant influence on c-myc mRNA levels alone (Figs. 1 and 3). The results are summarized in Fig. 7. Trifluoperazine, 50 \(\mu\)M, calmidazolium, 5 \(\mu\)M, and CsA, 200 nm, failed to block the Epo effect on the c-myc mRNA levels.

Effect of Cyclosporin A on the Induction of Hemoglobin Synthesis in ELM-I-1 and F4-6 Cells—In order to study the effects of the inhibitors tested on the differentiation status of ELM-I-1 cells, hemoglobin production was determined at 72 h after drug treatments. Longer incubations with calmodulin antagonists proved to be highly toxic for the cells. On the other hand, CsA was tolerated by the cells without decrease in cell viability. We tested the effect of CsA, 200 nm on the hemoglobin synthesis in ELM-I-1 cells induced by Epo, 0.5 unit/ml, or by the [Ca\textsuperscript{2+}]-increasing agent cyclopiazonic acid, 2.5 and 5 \(\mu\)M. Cyclopiazonic acid was shown to increase hemoglobin production in Friend erythroleukemia cells with lower toxicity (8). Epo induced a 5–6-fold increase in hemoglobin production, while cyclopiazonic acid proved to be less effective with a 2–3-fold increase in hemoglobin synthesis. Similarly to the effects on c-myc mRNA levels (Figs. 3 and 7), CsA antagonized the Ca\textsuperscript{2+}-mediated increase in hemoglobin production, while the Epo-induced hemoglobin synthesis was not affected (Table I). CsA also inhibited the decrease in cell viability in the cyclopiazonic acid-treated cultures.

Since cyclopiazonic acid induced only a moderate hemoglobin production in ELM-I-1 cells, experiments were performed also with the Friend erythroleukemia cell line F4-6 (14) in which [Ca\textsuperscript{2+}]-increasing agents induced hemoglobin production more effectively (8). A maximum in hemoglobin production in this cell line was observed after 96-h incubation. The effect of cyclopiazonic acid, 2.5 and 5 \(\mu\)M, was compared in the Epo-insensitive F4-6 cells with the strong chemical inducer of differentiation, Me\textsubscript{2}SO, 1.5% (Table II). The results show that the cyclopiazonic acid-induced hemoglobin production and decrease in cell viability was antagonized by CsA, 200 nm. On the other hand, CsA had no effect on the hemoglobin production and decrease in cell viability induced by Me\textsubscript{2}SO. The effects of the same drug treatments on the c-myc mRNA levels was comparatively studied in F4-6 cells at 3 h. CsA antagonized the decrease in c-myc mRNA by the [Ca\textsuperscript{2+}]-increasing agent cyclopiazonic acid, while the strong suppression of c-myc mRNA levels by Me\textsubscript{2}SO treatment was not inhibited (data not shown).

Lack of Erythropoietin-induced Changes in [Ca\textsuperscript{2+}]—In ELM-I-1 Cells—Previously we demonstrated the development of Ca\textsuperscript{2+} signals in F4-6 cells with cyclopiazonic acid and thapsigargin (8). In the present experiments the effect of Epo on Ca\textsuperscript{2+} was tested under similar experimental conditions in ELM-I-1 cells loaded with fura-2 fluorescent calcium indicator. As documented in Fig. 8, in unstimulated ELM-I-1 cells an approximately 110 nm resting cytoplasmic Ca\textsuperscript{2+} level could be measured in the presence of 0.5 mm free external Ca\textsuperscript{2+}. Treatment of the cells with repeated addition of Epo (2 units/ml, 10 units/ml, 50 units/ml) did not induce an increase in [Ca\textsuperscript{2+}], during the 20-min treatment period. In spite of the lack of an effect by Epo, 2 nm thapsigargin induced a rapid enhancement in [Ca\textsuperscript{2+}], (to approximately 450 nm) in the same ELM-I-1 cells. Similar results were obtained when the external Ca\textsuperscript{2+} concentration was increased up to 2.5 mm, and no differences were seen when human or mouse recombinant Epo preparations were used (data not shown).

**DISCUSSION**

In accordance with previous studies in Friend erythroleukemia cells (7, 8), the present results demonstrate a rapid sup-
expression of c-myb mRNA levels by [Ca\textsuperscript{2+}]_i-increasing agents in the Epo-sensitive murine erythroleukemia cell line ELM-I-1 (12). The Ca\textsuperscript{2+}-induced down-regulation of c-myb expression could be inhibited with the CaM antagonists, trifluoperazine and calmidazolium (27), as well as with CsA, an inhibitor of the Ca\textsuperscript{2+}/CaM-dependent serine/threonine-specific protein phosphatase, PP2B, or calcineurin (31, 32). CsA inhibited the Ca\textsuperscript{2+} effect on c-myb mRNA with the same concentration dependence as the calcineurin phosphatase activity (Figs. 3B and 4). On the other hand, FK506, another immunosuppressant and well-characterized inhibitor of calcineurin (31, 32), was unable to inhibit the Ca\textsuperscript{2+}-induced c-myb mRNA decrease and PP2B activity in ELM-I-1 cells. Similar differences in the effects of CsA and FK506 were previously observed in other cell lines. In mouse bone marrow-derived progenitor mast cells, CsA inhibited calcineurin activity effectively (IC\textsubscript{50} = 8 nM), while FK506 was without effect at concentrations up to 1000 nM (34). Resistance to FK506 was associated with a deficiency in FK506-binding immunophilin, FKBP12. A decreased sensitivity of PP2B to FK506 relative to CsA has also been observed in rat pancreatic acinar cells (35). Therefore, ELM-I-1 cells used in the present study represent an additional cell line with a lack of sensitivity of calcineurin to the inhibitory action of FK506. It would be of interest to determine whether this is also a characteristic of human erythroid cells.

The effect of CaM antagonists and the close correlation of the effect of immunosuppressants on the c-myb mRNA changes and calcineurin phosphatase activity strongly suggest that the Ca\textsuperscript{2+}-induced down-regulation of c-myb expression is mediated by calcineurin. Calcineurin has been implicated in the regulation of gene expression in different cell systems. In T lymphocytes, calcineurin was identified as a key enzyme in the T cell receptor-mediated signal transduction pathways playing a positive regulatory role in the transcription of interleukin-2 or interleukin-4 genes (36–38). In a pancreatic islet cell line, calcineurin is involved in the cAMP response element (CRE)-mediated induction of glucagon gene transcription after membrane depolarization (39). Both positive and negative signaling functions of calcineurin have been demonstrated in the transcriptional regulation of immediate early genes in PC12 cells (40). In this cell line, calcineurin mediates the activation of NGFI-B and inhibition of NGFI-A transcription in response to Ca\textsuperscript{2+} signals in synergism or antagonism with Ca\textsuperscript{2+}/CaM-dependent protein kinases. The present results with ELM-I-1 cells suggest a negative regulatory role of calcineurin in the c-myb expression, similarly to the NGFI-A expression in PC12 cells (40). However, experiments in ELM-I-1 cells with KN-62, an inhibitor of Ca\textsuperscript{2+}/CaM-dependent protein kinases (28–30), provided no evidence that a Ca\textsuperscript{2+}/CaM-dependent kinase is involved in early Ca\textsuperscript{2+}-induced changes in c-myb mRNA levels.

The target of action of calcineurin in the regulation of c-myb expression remains to be elucidated. In T lymphocytes, NF-AT-like transcription factors operate as downstream components of the calcineurin pathway (36, 38). In ELM-I-1 cells, dephosphorylation by calcineurin may result in activation of negative regulatory proteins or inactivation of protein factors necessary for the maintenance of a high level of c-myb mRNA in these cells. Since calcineurin is known to activate PP1 in certain tissues via a protein phosphatase cascade (41–43), PP1 could also mediate these effects of calcineurin. CREB, a well-characterized transcription factor, which binds to CRE in the promoter region of cAMP- or Ca\textsuperscript{2+}-responsive genes (44, 45), can be efficiently dephosphorylated by PP2B in vitro (30) and by PP1 in vivo (46, 47). However, there is no indication that c-myb expression is regulated by a CREB-dependent mechanism. The murine c-myb gene has a weak promoter (48) and transcriptional elongation appears to be the main target of regulation (49, 50). A transcriptional arrest mechanism operates in the first intron of the mouse c-myb gene, and sequence-specific protein binding in this region could play an important role in the regulation of c-myb expression (51).

The physiological regulator of erythropoiesis, Epo, has been shown to induce a rapid down-regulation of c-myb mRNA levels in various murine erythroleukemia cell lines (2–5). We now
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Ca²⁺ and Erythropoietin (Epo) effects on c-myc expression and hematopoiesis remain undefined. Under the present experimental conditions, an increase in [Ca²⁺]i by Epo could not be demonstrated. This observation is consistent with similar negative results from other laboratories (reviewed in Ref. 9). However, several other studies, which report on the [Ca²⁺]i-increasing activity of Epo (9–11), suggest that under certain conditions, Epo receptors may generate Ca²⁺ signals. Miller et al. (52) have demonstrated that Epo stimulates a rise in [Ca²⁺]i in single BFU-E-derived erythroblasts at specific stages of differentiation. Accordingly, the Epo effect on [Ca²⁺]i may depend on the cell line investigated and on its developmental stage. These results leave the possibility open that Epo can influence c-myc expression and hemoglobin synthesis also via a Ca²⁺/CaM-dependent pathway. This could act synergistically to the Ca²⁺/CaM-independent Epo signaling. In fact, our recent experiments indicate that okadaic acid above 100 nM inhibits also the Ca²⁺/CaM and calcineurin.

However, a common characteristic of the action of Epo and [Ca²⁺]i-increasing agents is the rapid down-regulation of c-myc mRNA without similar effect on the c-myc expression (2–5, 7, 8). Patel et al. (5) have demonstrated that okadaic acid, 100–400 nM, inhibits the Epo effect on c-myc mRNA levels in Rauscher erythroleukemia cells, suggesting a role of PP2A and/or PP1. Dephosphorylation of the same regulator protein(s) by Ca²⁺/CaM-dependent or -independent serine/threonine-specific protein phosphatases may represent a common mechanism of the action of Epo and [Ca²⁺]i-increasing agents in the down-regulation of c-myc expression. Alternatively, an activation of PP1 by Epo or by [Ca²⁺]i-increasing agents via a PP2B/PP1 cascade (41–43) could commonly mediate Epo or Ca²⁺ signals to c-myc. In fact, our recent experiments indicate that okadaic acid above 100 nM inhibits also the Ca²⁺ effect on c-myc mRNA levels in ELM-I-1 cells (data not shown). The relative high inhibitory concentrations needed may point to a role of PP1, which is less effectively inhibited by this compound than PP2A (42). Studies are in progress in our laboratory to test this possibility.

The early down-regulation of c-myc expression has been suggested to be a prerequisite for either Epo-initiated or chemically induced erythroid differentiation in murine erythroleukemia cells (2, 4). Previously, we reported that the Ca²⁺-mediated transient suppression of c-myc mRNA levels is followed by induction of globin mRNA and hemoglobin synthesis in Friend erythroleukemia cells, line F4-6 (7). The present experiments show that prevention of the early decrease in c-myc mRNA by CsA is correlated with an inhibition of hemoglobin production in ELM-I-1 or F4-6 cells incubated in the presence of the [Ca²⁺]i-increasing agent, cyclopiazonic acid (Tables I and II). These results provide further evidence that down-regulation of c-myc expression is associated with the initiation of hemoglobin synthesis in erythroid precursor cells (6). However, cyclopiazonic acid proved to be less active than Epo in increasing hemoglobin synthesis in ELM-I-1 cells. Toxic effects may attenuate the differentiation-inducing potency of the [Ca²⁺]i-increasing agent during longer incubation periods. MeSO also proved to be a rather weak inducer of hemoglobin synthesis in this cell line (12).

On the other hand, the experiments also demonstrate that the Epo-induced down-regulation of c-myc expression and stimulation of hemoglobin synthesis in ELM-I-1 cells occurred independently of Ca²⁺/CaM and calcineurin. Therefore, the relevance of the Ca²⁺ effects on c-myc expression and hemoglobin synthesis for the physiological signal pathway of erythroid differentiation remains undefined. Under the present experimental conditions, an increase in [Ca²⁺]i by Epo could not be demonstrated. This observation is consistent with similar negative results from other laboratories (reviewed in Ref. 9). However, several other studies, which report on the [Ca²⁺]i-increasing activity of Epo (9–11), suggest that under certain conditions, Epo receptors may generate Ca²⁺ signals. Miller et al. (52) have demonstrated that Epo stimulates a rise in [Ca²⁺]i in single BFU-E-derived erythroblasts at specific stages of differentiation. Accordingly, the Epo effect on [Ca²⁺]i may depend on the cell line investigated and on its developmental stage. These results leave the possibility open that Epo can influence c-myc expression and hemoglobin synthesis also via a Ca²⁺/CaM-dependent pathway. This could act synergistically to the Ca²⁺/CaM-independent Epo signaling. In fact, the Ca²⁺ effects on c-myc expression and hemoglobin synthesis suggest the possibility of a pharmacological influence on erythroid differentiation. Further studies, especially with human erythroid precursor cells, are necessary to explore these possibilities.
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