Erythropoietin (Epo) stimulation of erythroid cells results in the activation of several kinases and a rapid induction of c-myc expression. Protein kinase C is necessary for Epo up-regulation of c-myc by promoting elongation at the 3'-end of exon 1. PKCε mediates this signal. We now show that Epo triggers two signaling pathways to c-myc. Epo rapidly up-regulated Myc protein in Ba/F3-EpoR cells. The phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 blocked Myc up-regulation in a concentration-dependent manner but had no effect on the Epo-induced phosphorylation of ERK1 and ERK2. LY294002 also had no effect on Epo up-regulation of c-fos. MEK1 inhibitor PD98059 blocked both the c-myc and the c-fos responses to Epo. PD98059 and the PKC inhibitor H7 also blocked the phosphorylation of ERK1 and ERK2. PD98059 but not LY294002 inhibited Epo induction of ERK1 and ERK2 phosphorylation in normal erythroid cells. LY294002 blocked transcription of c-myc at exon 1. PD98059 had no effect on transcription from exon 1 but, rather, blocked Epo-induced c-myc elongation at the 3'-end of exon 1. These results identify two Epo signaling pathways to c-myc, one of which is PI3K-dependent operating on transcriptional initiation, whereas the other is mitogen-activated protein kinase-dependent operating on elongation.

Erythroid progenitor cell growth and differentiation are regulated by the hematopoietic growth factor erythropoietin (Epo). The interaction of Epo with its cognate receptor (EpoR) initiates a cascade of signaling pathways that mediate Epo's growth-promoting, anti-apoptotic, and differentiation-inducing actions. Previously, we reported that Epo up-regulates c-myc as a primary (immediate/early) response gene via a protein kinase C (PKC)-dependent pathway (1, 2) and that this PKC-dependent signal increased c-myc expression by reducing transcriptional attenuation (arrest) at the 3'-end of exon 1, thereby inducing transcriptional elongation (3). Later, we showed that the PKCε isoform mediates this signal and that down-regulation of PKCε inhibits Epo's growth-promoting action but not its induction of β-globin expression (differentiation) (4).

We have now investigated Epo up-regulation of c-myc further. We report that Epo's PKC-dependent signal to the transcriptional attenuation site in exon 1 of c-myc utilizes the MEK/ERK pathway and, simultaneously, that this pathway leads to up-regulation of c-fos. In addition, we have discovered that Epo independently up-regulates c-myc expression by increasing transcriptional initiation through a phosphoinositide 3-kinase (PI3K)-dependent pathway.

EXPERIMENTAL PROCEDURES

Cell Culture and Epo Treatment—Two types of Epo-responsive cells were used in these studies. First, Ba/F3 cells stably transfected with the human EpoR cDNA (Ba/F3-EpoR cells, generous gift of M. Showers) (5–9) were maintained in a humidified atmosphere of 95% air/5% CO₂ at 36.5 °C in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone Laboratories) and 1 unit of Epo/ml (Elaxen Pharmaceuticals). Cells were incubated overnight in the absence of fetal bovine serum and Epo, and then were treated with 5 units of Epo/ml for specified times. In some experiments, after overnight serum-free incubation, cells were incubated in the presence of specified concentrations of the PI3K inhibitor LY294002 (10) or of the MEK inhibitor PD98059 (11–13) (Sigma-Aldrich) for 10 min. Then 5 units of Epo/ml was added, and the incubation was continued for an additional 40 min. The cells were harvested by centrifugation for mRNA preparation, nuclear extraction, and/or Western blotting. Second, normal erythroid cells were obtained from the spleens of phenylhydrazine-treated mice (2). B6C3F1 mice less than 10 weeks old were injected subcutaneously on day 1 with a sterile solution of 2 mg of phenylhydrazine/ml in phosphate-buffered saline to achieve a dose of 60 mg/kg. The injections were repeated on day 2, and the mice were killed on day 5 by cervical dislocation. The enlarged spleens were excised and pressed between two microscope slides, and the cells were suspended in cold Eagle's medium, alpha modification (α-MEM). A single cell suspension was prepared by consecutive passage through 18-gauge and 23-gauge needles. The cells were washed and resuspended in α-MEM (10⁶ cells/ml). An equal volume of cold ammonium chloride solution (0.83% in 0.01 M Tris-HCl, pH 7.5) was added to lyse the erythrocytes. After 10 min on ice, the cells were washed again and plated at 1 × 10⁶/ml in 37 °C α-MEM with 10% fetal bovine serum for 4 h. Then the cells were incubated in the absence or presence of specified concentrations of LY294002 of PD98059 for 10 min followed by the addition of 12 units of Epo/ml. Incubation was continued for 50 min. The cells were harvested by centrifugation for nuclear extraction and Western blotting. HeLa cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium/10% fetal bovine serum. They were incubated overnight in the absence of serum followed by incubation in the absence or presence of 100 ng EGF/ml for 10 min.

Western Blot Analysis—Western blot analyses were carried out either on whole cell lysates or on nuclear extracts. Whole cell lysates were prepared as follows. 5 × 10⁶ cells were lysed in 250 μl of 1 × SDS sample buffer (New England BioLabs). The cell lysate was sonicated for 15 s and heated in boiling water for 5 min. Samples were centrifuged at 12,000 rpm for 5 min. 20 μl of sample were subjected to 8% SDS-PAGE and then transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore). The membrane was washed with 25 ml of 1× Tris-buffered saline (20 mM Tris-HCl, 140 mM NaCl, pH 7.6) then
incubated in 15 ml of blocking buffer (1× Tris-buffered saline, 0.1% Tween 20, 5% w/v nonfat dry milk) for 1 h at room temperature. The membrane was incubated with a 1:1000 dilution of the specified primary antibody (New England Biolabs) in 10 ml of blocking buffer with gentle shaking overnight at 4 °C. After three 5-min washes in blocking buffer, the membrane was incubated with 1:1000 alkaline phosphatase-conjugated secondary antibody (New England Biolabs) in 10 ml of blocking buffer with gentle shaking for 1 h at room temperature. Proteins were detected using a CDP-Star Western blotting kit (New England Biolabs). For reprobing of membrane with a second primary antibody, the membrane was incubated in strip buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) for 30 min at 50 °C, then washed and blocked as above. Nuclear extracts were prepared using NE-PER extraction reagents (Pierce) per the manufacturer’s instructions. Protein amounts in the bands were quantified by densitometric analysis of scanned images using Gel-Pro Analyzer, version 3.1 (Media Cybernetics).

**RNA Isolation and RT-PCR Analysis**—10×10⁶ cells were collected by centrifugation at 2000 × g for 5 min. After carefully removing the supernatant, cells were lysed by trituration in 1 ml of TRIzol reagent (Life Technologies). The lysed samples were incubated for 5 min at room temperature, and 0.2 ml of chloroform was added. After shaking and incubating for 3 min, samples were centrifuged for 15 min at 4 °C, and 0.5 ml of the aqueous phase containing RNA was collected. Isopropyl alcohol (0.5 ml) was added to the aqueous phase to precipitate RNA. The mixture was incubated for 10 min at room temperature and then centrifuged for 10 min at 4 °C. After being washed with 1 ml of 75% ethanol and dried under vacuum, the RNA was dissolved in 400 μl of distilled H₂O.

PCR primers for mouse c-myc, c-fos, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were derived from the published sequences. The sequences of the primers and their position in the gene sequence are as follows: c-myc, 5'-primer, 5'-CTCTGCTCCTGTAAGGACACGT-TCGCC (57–84 nucleotides related to the promoter 1 initiation site); 3'-primer within exon 1, 5'-TCAGAGAACCTGAGCTACTGGCAAGCC-AGAGAGAC (472–508); 3'-primer within exon 2, 5'- GTGAGTGAGG- TTTCACGTTGAGGGGCATCGTCGTGGC (2104–2141); c-fos, 5'-primer, 5'-CTCTGTCACCCACTGTGGCTC (224–244); 3'-primer, 5'-CTTTCA- GCAGATTTCACCTC (540–561); GAPDH, 5'-primer, 5'- CACATGCTGCTGACCACGAAG (527–550); 3'-primer, 5'-GTCACACACCCTGTTGCTGTTACCG (947–971). First-strand cDNA was synthesized using oligo(dT)₁₆ primer by incubating 1 μg of cellular RNA in 20 μl of reverse transcription reaction containing 1 × PCR buffer II, 2.5 units of murine leukemia virus reverse transcriptase (PerkinElmer Life Sciences), 1 mM dNTP, and 5 mM MgCl₂ at 42 °C for 15 min. PCR amplification was carried out in 1× PCR buffer II with 0.0125 unit of Taq DNA polymerase (PerkinElmer Life Sciences) and 2 mM MgCl₂ for 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s in a PerkinElmer Life Sciences thermal cycler GeneAmp PCR system 2400. As an internal control for Epo-induced changes in c-myc and c-fos expression, GAPDH primers flanking a 444-bp GAPDH cDNA fragment were included in each PCR reaction. In initial experiments, the GAPDH primers were included in the same reaction tube as were the c-myc and c-fos primers. These experiments demonstrated no change in GAPDH product upon Epo stimulation, consistent with our previous work (1, 2, 4, 14). Because of the abundance of the GAPDH product compared with those of c-myc or c-fos, in later studies such as those depicted in Figs. 11 and 12 (see below), we carried out the GAPDH reaction in a separate PCR tube but simultaneously with the c-myc or c-fos PCR reaction. The PCR products were resolved in a 1.5% agarose gel and visualized with ethidium bromide staining. Alternatively, RT-PCR was performed with c-myc gene exon 1 or exon 2 3'-primer for reverse transcription instead of using oligo(dT)₁₆, 57 μl of reaction mixture containing 3 μg of cellular RNA, 1× PCR buffer II, 2.5 units of murine leukemia virus reverse transcriptase (PerkinElmer Life Sciences), 1 mM dNTP, and 5 mM MgCl₂ was aliquoted into three tubes each containing 100 pmol (1 μl) of 3'-primer of c-myc exon 1, exon 2, or GAPDH, respectively. The reverse transcription reaction was carried out at 42 °C for 15 min. After the reverse transcription reaction, PCR reaction mixture containing 1× PCR buffer II with Taq DNA polymerase, 2 mM MgCl₂, and 5'-primer of c-myc, c-fos, or GAPDH, respectively, was aliquoted into the reverse transcription reaction mixture. PCR amplification was carried out as above. After reverse transcription, a series of reactions (1, 2, 4, 8, and 16 μl) was used for PCR amplification with GAPDH primer to confirm that the relationship between the amount of template and the yield of PCR product was linear.

**RESULTS**

Western blot analysis showed that Epo up-regulates c-myc expression at the protein level in BaF3-EpoR cells. Cells were exposed to 5 units of Epo/ml for specified times. Total cells lysates were prepared, and equal amounts of protein were subjected to SDS-PAGE and Western blotting with anti-Myc antibody. In the absence of added Epo, the cells expressed low levels of Myc1 and Myc2 of ~67 and 64 kDa, respectively (Fig. 1, upper panel). Myc1 was not detected in all experiments. After 30 min of exposure to Epo, Myc2 levels increased 4.2-fold. The amount seen in the absence of Epo and continued to do so, reaching levels of 15- and 18-fold at 45 and 60 min of Epo treatment, respectively. Epo-induced increases in Myc protein were observed in several additional experiments. The magnitude of the increases varied somewhat from experiment to experiment. To demonstrate equal protein loading of the gel, the membrane was stripped and reprobed with antibody to the p44/42 MAP kinases ERK1 and ERK2 (Fig. 1, bottom panel). ERK1 and ERK2 levels were unchanged during the same Epo treatment period.

We showed previously that Epo’s signal to c-myc requires PKC (1–4). We have now discovered that Epo’s signal to c-myc also requires PI3K and the MEK/ERK pathway. BaF3-EpoR cells were incubated in the absence or presence of 5 units of Epo/ml for 40 min and in the absence or presence of specified concentrations of the PI3K inhibitor LY294002 (10) (see “Experimental Procedures”). Cell lysates were prepared, and equal amounts were subjected to SDS-PAGE and Western blotting with anti-Myc antibody or with anti-ERK antibody. LY294002 inhibited Epo up-regulation of Myc expression completely in a concentration-dependent manner (Fig. 2, top panel). As a control experiment (Fig. 2, bottom panel), probing with anti-ERK antibody revealed no change in ERK1/ERK2 levels. Similarly, incubation of Epo-treated cells in the absence or presence of MEK (MAPK kinase) inhibitor PD98059 (11–13) resulted in a concentration-dependent inhibition of Myc up-regulation (Fig. 3). These results, along with our previously published data (1–4), suggested that Epo regulation of c-myc expression requires PKC, PI3K, and the MEK/ERK pathway.
We confirmed that LY294002 and PD98059 had each down-regulated c-myc mRNA levels in BaF3-EpoR cells by reverse transcription-PCR (RT-PCR) (Fig. 4, A and B). Total cellular RNA from cells incubated in the absence or presence of 5 units of Epo/ml for 40 min and were processed as described in the legend to Fig. 1. Myc amounts were quantified as Integrated Optical Density units and were normalized to the Myc band observed in the absence of Epo.

We also demonstrated the specificity of this effect on c-myc expression by examining c-fos (Fig. 5, A and B). BaF3-EpoR cells were incubated in the absence or presence of 5 units of Epo/ml for 40 min and in the absence or presence of specified concentrations of LY294002 or PD98059. Epo treatment of the cells resulted in the appearance of a 338-bp c-fos product. LY294002 did not inhibit Epo up-regulation of c-fos (Fig. 5A), in contrast to its inhibition of Epo up-regulation of c-myc, indicating that Epo’s signal to c-fos does not require PI3K. However, PD98059 did inhibit Epo up-regulation of c-fos in a concentration-dependent manner (Fig. 5B). This result was expected, because c-fos has been described to be downstream of the MEK/ERK signaling pathway (see “Discussion”). Thus, in this BaF3-EpoR cell system, MEK/ERK are not downstream of PI3K (see “Discussion”). This result implies that the LY294002-inhibited PI3K pathway to c-myc and the PD98059-inhibited MEK/ERK pathway to c-myc are independent and that both are independently necessary for Epo up-regulation of c-myc.

We showed further that the MEK/ERK pathway is not downstream of PI3K in BaF3-EpoR cells by studying Epo-induced phosphorylation of ERK (Fig. 6). Cells were incubated in the absence or presence of 5 units of Epo/ml for 40 min and in the absence or presence of specified concentrations of LY294002 or PD98059. Nuclear extracts were prepared, and equal amounts were subjected to SDS-PAGE followed by Western blotting with anti-phospho-ERK antibody or with anti-ERK antibody. In the absence of Epo, the cells contained trace equally using RNA from cells incubated under each of the conditions, confirming the specificity of the LY294002 and PD98059 effects.
amounts of phospho-ERK1 and phospho-ERK2 (Fig. 6, top left and top center panels). Treatment of the cells with Epo resulted in a marked increase in phospho-ERK1 and a significant although somewhat lesser increase in phospho-ERK2 (Fig. 6, top left and top center panels, second lanes, Epo). This preferential increase in phospho-ERK1 occurred even though the abundance of ERK2 protein exceeded that of ERK1 (Fig. 6, bottom left and bottom center panels). Epo had no effect on the levels of ERK1 or ERK2 protein (Fig. 6, bottom left and bottom center panels). Epo-induced phosphorylation of ERK1 and ERK2 was not inhibited by LY294002 (Fig. 6, top left panel) but was almost completely inhibited in a concentration-dependent manner by PD98059 (Fig. 6, top center panel). The levels of ERK1 and ERK2 protein were unchanged under all conditions (Fig. 6, bottom left and bottom center panels).

The unexpected preferential increase in phospho-ERK1 over phospho-ERK2 in Epo-induced BaF3-EpoR cells, despite the predominant abundance of ERK2 protein, was seen repeatedly in several experiments. To prove that this finding was not an artifact, we performed the following control experiment. We grew HeLa cells, which express the EGF receptor, the stimulation of which induces ERK phosphorylation (15, 16), and stimulated them with 100 ng EGF/ml. Nuclear extracts were prepared and were analyzed on the same SDS-PAGE run as were the BaF3-EpoR cells extracts (Fig. 6, right top and bottom right panels). Nuclear extracts of HeLa cells incubated in the absence of added EGF contained modest amounts of phospho-ERK1 and phospho-ERK2. Importantly, the phospho-ERK1 and phospho-ERK2 were of equal abundance. Incubation of the cells in the presence of EGF induced an equal increase in the abundance of both phospho-ERK1 and phospho-ERK2. Total ERK2 protein was slightly more abundant than ERK1 in the HeLa nuclear extracts. ERK1 and ERK2 from HeLa cells, which are human in origin, migrated consistently as a more closely spaced doublet than did ERK1 and ERK2 from BaF3-EpoR cells, which are murine in origin.

As a control to demonstrate the ability of LY294002 to inhibit PI3K in the BaF3-EpoR cell experiments, we stripped the blot shown in Fig. 6 and reprobed it with anti-phospho-Akt antibody (Fig. 7). Akt (protein kinase B) is a downstream target of the PI3K-dependent signaling pathway, being phosphorylated by PDK1 (17). Nuclear extracts of cells incubated in the absence of Epo contained a small amount of phospho-Akt (Fig. 7, top and center panels). Incubation in the presence of Epo for 40 min induced a 2.8- to 3.1-fold increase in phospho-Akt. LY294002 treatment resulted in a concentration-dependent decrease in phospho-Akt to baseline levels, confirming that LY294002 was active in blocking PI3K in these experiments (Fig. 7, top panel). PD98059 had no effect on Epo-induced Akt phosphorylation, as expected (Fig. 7, center panel). We had shown previously that PKC inhibitors block c-myc transcriptional elongation similar to that seen in the present study with the MEK inhibitor PD98059 (3). This suggested that PKC might be upstream of MEK in the Epo signaling cascade in BaF3-EpoR cells. To test this possibility, we incubated cells in the absence or presence of 5 units of Epo/ml for 40 min and with specified concentrations of the PKC inhibitor H7. As seen in Fig. 8, top panel, Epo induced an increase in phospho-ERK1 and phospho-ERK2. Addition of H7 resulted in a concentration-dependent decrease in Epo-induced phospho-ERK1 and phospho-ERK2, supporting the hypothesis that PKC is upstream of MEK in this pathway.

The results presented above indicate that PI3K is not upstream of the MEK/ERK pathway in the Epo signal cascade of BaF3-EpoR cells. This finding is in apparent contradiction to two other studies that employed different cells to investigate Epo signal transduction. In the first study, Klingmüller et al. (18) isolated fetal liver (erythroid) cells from Epo−/− mice and introduced a mutant EpoR into the cells by retroviral gene transfer. The mutant EpoR contained only one tyrosine (Tyr-479) in its cytoplasmic domain, the other seven having been replace by phenylalanine. Exposure of the cells to Epo triggered association of PI3K with the mutant receptor and activation of MAPK, leading to proliferation and differentiation of the cells in vitro, thus placing MAPK downstream of PI3K in these cells. In the second study, Sui et al. (19) studied purified human “erythroid colony-forming cells.” Treatment of these cells with Epo resulted in a very modest increase in phospho-ERK1 and phospho-ERK2. The increase was apparently blocked by pretreatment either with 100 μM PD98059 or with 0.5 μM wortmannin. To determine if our results on ERK phosphorylation are unique to the BaF3-EpoR cell system, we carried out studies using normal erythroid cells isolated from the spleens of phenylhydrazine-treated mice (2, 20, 21). As seen in Fig. 9, normal erythroid cells incubated in the absence of Epo contained a modest amount of phospho-ERK1 and a trace amount of phospho-ERK2 (Fig. 9, top). Incubation of the cells in the

Fig. 5. Effect of PI3K inhibitor LY294002 (A) or by MEK inhibitor PD98059 (B) on erythropoietin-induced c-fos mRNA in BaF3-EpoR cells. Results of RT-PCR of cellular RNA from cells incubated in the absence or presence of 5 units of Epo/ml and in the presence of specified concentrations of inhibitor. M = markers. Note the appearance of the 338-bp c-fos product in cells treated with Epo (Epo). LY294002 had no apparent effect on c-fos mRNA; however, increasing concentrations of PD98059 resulted in disappearance of the c-fos transcript. GAPDH, co-amplified GAPDH product. Fos PCR product amounts were first quantified as reciprocal Integrated Optical Density units and then were normalized to the product seen in lane EPO/H11001, which were set as 100%. The values shown should be considered as semiquantitative.
The presence of Epo resulted in a 3.9-fold increase in total phospho-ERK1/2, with phospho-ERK1 in significantly greater abundance. The PI3K inhibitor LY294002 did not inhibit this Epo-induced increase in phospho-ERK1/2. In contrast, the MEK inhibitor PD98059 inhibited this increase in phospho-ERK1/2 almost completely. These results mirror those shown above that we obtained using BaF3-EpoR cells and indicate that PI3K is not upstream of the MEK/ERK pathway in the Epo signal cascade of these normal erythroid cells. We confirmed the action of LY294002 in these cells by stripping the membrane shown in Fig. 9 and reprobing it with anti-phospho-Akt antibody (Fig. 10, top panel). Untreated normal erythroid cells contained a small amount of phospho-Akt. Epo induced a 2.7-fold increase. LY294002 inhibited Akt phosphorylation dramatically. In contrast PD98059 had only a minor effect on phospho-Akt at the highest concentration employed (50 μM).

FIG. 6. Effect of PI3K inhibitor LY294002 (left panels) or MEK inhibitor PD98059 (center panels) on erythropoietin induction of ERK phosphorylation. Experiments were carried out on nuclear extracts (see “Experimental Procedures” and “Results”). Incubation of the cells in the presence of Epo resulted in a substantial increase in phospho-ERK1 and a somewhat lesser increase in phospho-ERK2 (P-ERK1/2, top left and top center panels). Addition of LY294002 did not appear to alter this (top left panel). However, addition of PD98059 inhibited this Epo-induced ERK1 and ERK2 phosphorylation markedly (top center panel). ERK protein levels were unchanged under all conditions (bottom left and bottom center panels). Note the preferential phosphorylation of ERK1 despite greater abundance of ERK2 protein. Right panels, top and bottom, show essentially equal EGF-induced phosphorylation of ERK1 and ERK2 in HeLa cells, in contrast to the preferential phosphorylation of ERK1 in BaF3-EpoR cells (see “Results”).

FIG. 7. Effect of PI3K inhibitor LY294002 on erythropoietin-induced Akt phosphorylation in BaF3-EpoR cells. Epo induced a significant increase in phospho-Akt. LY294002 inhibited this increase (top panel), whereas MEK inhibitor PD98059 had no effect (center panel). An equivalent amount of ERK1/2 total protein in each lane confirms equal loading of the gel (bottom panel).

FIG. 8. Effect of PKC inhibitor H7 on erythropoietin-induced ERK phosphorylation in BaF3-EpoR cells. Cells were incubated in the absence or presence of specified concentrations of H7 for 30 min and then in the presence of 5 units of Epo/ml for an additional 40 min. Experiments were carried out on nuclear extracts (see “Experimental Procedures” and “Results”). H7 markedly inhibited Epo-induced phospho-ERK1 and phospho-ERK2 (top panel). There was no change in total ERK protein (bottom panel).
different mechanisms operating at two different sites on the gene. We showed this by carrying out RT-PCR experiments that detected either c-myc exon 1 expression (by amplifying a product between nucleotides 54 and 508 of the gene), thereby providing a measurement of transcriptional initiation, or that detected expression of c-myc exons 1 and 2 (by amplifying a product between nucleotides 54 and 2141 of the gene), thereby measuring elongation. As seen in Figs. 11A and 12A, treatment of cells with Epo resulted in the appearance of a 451-bp RT-PCR product, consistent with increased expression of exon 1 mediated by an increase in transcriptional initiation. LY294002 inhibited this increased expression of exon 1 in a concentration-dependent manner (Fig. 11A). This inhibition also resulted in decreased expression of exon 1/2 (Fig. 11B), consistent with reduced elongation due to increased transcriptional attenuation within exon 1. PD98059 did not inhibit Epo up-regulation of exon 1 by increased transcriptional initiation (Fig. 12A). However, PD98059 did inhibit the increased expression of exon 1/2 (Fig. 12B), consistent with reduced elongation due to increased transcriptional attenuation within exon 1. PD98059 had no significant effect on the control GAPDH product, confirming the specificity of the results seen in Fig. 12A and B (see Fig. 12C).

FIG. 9. Effect of PI3K inhibitor LY294002 or MEK inhibitor PD98059 on erythropoietin-induced ERK phosphorylation in normal murine erythroid cells. Cells were incubated in the absence or presence of 5 units of Epo/ml for 50 min and in the presence of specified concentrations of LY294002 or PD98059. Nuclear extracts were prepared and were subjected to SDS-PAGE and Western blotting as described above. Epo induced a greater increase in phospho-ERK1 than in phospho-ERK2 (top panel) despite the greater abundance of ERK2 protein (bottom panel). LY294002 had no effect on ERK phosphorylation. In contrast, PD98059 inhibited ERK phosphorylation almost completely.

FIG. 10. Effect of PI3K inhibitor LY294002 or MEK inhibitor PD98059 on erythropoietin-induced Akt phosphorylation in normal murine erythroid cells. Epo induced a significant increase in phospho-Akt. LY294002 inhibited this increase, whereas MEK inhibitor PD98059 had no effect (top panel). An equivalent amount of ERK1/2 total protein in each lane confirms equal loading of the gel (bottom panel).

FIG. 11. Effect of PI3K inhibitor LY294002 on erythropoietin up-regulation of c-myc expression. A, inhibition of erythropoietin-dependent increase in exon 1 expression. B, inhibition of erythropoietin-dependent increase in exon 1/2 expression. C, control GAPDH product. Myc, PCR product amounts were first quantified as reciprocal Integrated Optical Density units and then normalized to the product seen in lane EPO/LY294002, which was set as 100%. The values shown should be considered as semiquantitative.

DISCUSSION

The c-myc gene encodes a nuclear phosphoprotein, Myc, which forms heterodimers with the transcription factor Max and also interacts with several other proteins (22–24). These interactions are involved in the various biological activities of
Myc, including cell cycle regulation, transformation, and apoptosis. Normal expression of the c-myc gene is critical for regulated cell proliferation, whereas deregulated expression of c-myc is a frequent hallmark of neoplasia and is associated with cellular apoptosis. c-myc gene expression is controlled at multiple levels, including transcriptional initiation at several promoters, transcriptional elongation (transcriptional attenuation), and post-transcriptional regulation of mRNA stability (22–24).

c-myc was first implicated in erythroid cell growth and differentiation in studies of murine erythroleukemia cells treated with the chemical inducer dimethyl sulfoxide (MeSO) (25–28). MeSO treatment resulted in the biphasic down-regulation of c-myc transcript levels. Interestingly, transfection of these cells with a constitutively expressed c-myc resulted in inhibition of MeSO-induced differentiation. These results suggested that down-regulation of c-myc was essential for erythroid differentiation. However, other investigators used an Epo-sensitive cell line and demonstrated that, in contrast to the effect of MeSO on c-myc, Epo treatment up-regulated expression of the proto-oncogene concomitant with induction of differentiation (29). Although up-regulation of c-myc by Epo was not detected in studies of Epo-sensitive ELM-1-1 cells (30), it was detected in a novel J2E cell line (31) and was later shown also in erythroid cells derived from the spleens of Friend virus-infected mice, Epo up-regulated c-myc expression and that in culture antisense oligodeoxynucleotides to c-myc reduced human colony-forming unit-erythroid growth but not differentiation.

The present study demonstrates that Epo up-regulates c-myc expression in BaF3-EpoR cells by two independent signaling pathways. The data show that Epo’s signal to the transcriptional attenuation site in exon 1 (the signal dependent upon PKC) requires the MEK/ERK pathway. Activation of the MEK/ERK pathway by Epo in hematopoietic cells may occur by either of two mechanisms depending either upon Ras activation or upon PKC. Epo activation of p21Ras was first demonstrated in UT-7/EPO cells in which Epo induced an increase in exchange of GDP for GTP (33). Epo activation of p21Ras was also demonstrated in the human erythroleukemia cell line HEL in which stimulation with Epo induced a 5-fold increase in the amount of GTP bound to the endogenous p21Ras protein (34).

Later, Miura and colleagues (35) demonstrated Epo induction of Shc, ERK1, and ERK2 phosphorylation (35). Epo also increased association of Shc with Grb2 leading the authors to suggest that the carboxyl-terminal region of the EpoR may be important in activation of the MEK/ERK pathway mediated through Ras. In an interesting series of experiments, Xia and coworkers (36) showed that several cytokines that activate JAK2 kinase also stimulated Raf-1 kinase activity toward MEK. These investigators coexpressed Raf-1 and JAK2 in the human erythroleukemia cell line HEL, which proliferates in the presence of Epo and is induced to differentiate, Epo was not observed to activate the Ras pathway, whereas the pathway was activated by stem cell factor which proliferates in the presence of Epo and is induced to differentiate. Epo was not observed to activate the Ras pathway, whereas the pathway was activated by stem cell factor (38). Klingmuller et al. (18) studied erythroid progenitors from the livers of fetal Epo-receptor knock-out mice. After transfection with a mutant Epo-receptor, Epo treatment of these cells resulted in PI3K complexing with the receptor and activation of MEK, consistent with a PI3K-dependent signal to MEK. The discrepancy between these findings and our data is in all probability due, in part, to alternative technical approaches as well as differing cell backgrounds.

An earlier study by Carroll et al. (39) demonstrated that Epo induced Raf-1 activation and that Raf-1 was required for Epo-induced cell proliferation in HCD-57 and in FDC-P1/ER cells. In a later study (40), these investigators demonstrated PKC-
mediated phosphorylation of Raf-1 and suggested that PKC could promote hematopoietic cell growth by direct serine phosphorylation of Raf-1. In this regard, PKC-dependent activation of the MEK/ERK pathway has been shown in other cell types. For example, platelet-derived growth factor (PDGF) treatment of RAT-1 fibroblasts activates a phosphatiidylyceroline-specific phospholipase C resulting in the production of diacylglycerol and in the activation of PKCζ (41). PKCζ immunoprecipitates with and, apparently, phosphorylates and activates Raf-1 in this system. The action of VEGF on endothelial cells was shown to stimulate the Raf/MEK/ERK signal pathway without significantly activating Ras (42). Although dominant negative Ras mutants did not block the VEGF-dependent phosphorylation of MAPK, PKC-specific inhibitors were effective in markedly reducing the VEGF-dependent activation of the MEK/ERK pathway. Studies of neuronal cells, ERK activation by fibroblast growth factor and nerve growth factor were found to be dependent upon PKCζ. Also, EGF activation of MAPK was found to be dependent upon PKCζ, PI3K, and PDK1(Akt) in H19–7 hippocampal cells (43). Addition of a PKCζ inhibitor to L6 muscle cells transfected with the human insulin receptor had no effect on Ras activity but markedly inhibited insulin-induced Raf activation (44). In another interesting observation reported by Sui et al. (19), the PI3K inhibitor wortmannin alone inhibited MEK phosphorylation and MAPK activation induced by Epo in human peripheral blood erythroid colony-forming cells, implicating yet another upstream signaling element in erythropoietin activation of the MEK/ERK pathway.

As stated above, controversy exists regarding the precise role of the Raf/MEK/ERK pathway in erythroid cell growth and differentiation. Withdrawal of Epo from Epo-dependent HCD-57 cells resulted in a marked decrease in ERK phosphorylation and induction of apoptosis. Addition of Epo to these cells increased ERK phosphorylation. The authors concluded that Epo’s signal to ERK promotes cell survival (45). Nagata and Todokoro (46) also demonstrated increased ERK1 and ERK2 phosphorylation upon Epo stimulation of SKT6 cells. The MEK inhibitor PD98059 did not suppress differentiation. These results suggested that the MEK/ERK pathway leads to erythroid cell growth, along with its up-regulation of c-myc, and not to differentiation. Studies of other hematopoietic cells confirm Epo’s activation of ERK. Stimulation of UT7 cells resulted in increased phosphorylation of several proteins, including ERK1 and ERK2 (47). Epo stimulation of normal erythroid cells was also found to increase ERK1 phosphorylation (48), as did Epo stimulation of 32B/EpoR-wild type cells (49). Interestingly, this activation of ERK1 and ERK2 by Epo was enhanced by increased expression of CrkL adaptor protein, thereby connecting ERK activation with Ras. In a very recent study of HCD-57 cells, Epo was found to increase ERK1 and ERK2 phosphorylation (50). Again, inhibition of c-myc up-regulation with PD98059 inhibited ERK phosphorylation and blocked cell proliferation, closely linking the MEK/ERK pathway to Epo’s growth-promoting signal, a signal that we have now shown also up-regulates c-myc.

We have shown that Epo activation of the MEK/ERK pathway up-regulates c-fos expression in addition to its action on transcriptional elongation of c-myc. Epo up-regulation of c-fos was demonstrated several years ago in ELM-1-1 cells (30). This action of Epo on Epo-dependent cells was confirmed by Miura et al. (51) who demonstrated an up-regulation of c-fos, c-myc, and egr1. Studies from our laboratory utilizing Rauscher murine erythroleukemia cells, which are Epo-independent for growth but differentiate in response to it, did not detect an increase in c-fos mRNA levels but did document an increase in AP-1 activity by electrophoretic mobility shift assay, suggesting that a modification of Fos or Jun proteins was involved (52). Epo activation of AP-1 was also demonstrated by Bergelson et al. (53) who identified Tyr-343 and Tyr-464 of the EpoR as necessary for maximal activation and by Jacobs-Helber et al. (54) who found a role for AP-1 in Epo-dependent proliferation of HCD57 cells and in the apoptosis induced by Epo withdrawal. Further studies of transformed Epo-dependent cells demonstrated that the membrane proximal region of the EpoR is necessary and sufficient for the mitogenic action of the hormone along with JAK2 activation and induction of c-fos, c-myc, and other early response genes (55). In contrast to these observations, however, an examination of Friend virus-infected murine erythroblasts showed up-regulation of c-myc by Epo but did not demonstrate any increase in c-fos or c-jun transcript (32).

The data presented in this study show no inhibition of c-fos up-regulation by the PI3K inhibitor LY294002, but rather that the MEK/ERK pathway is upstream of c-fos. These findings are in agreement with results from other cell systems. For example, studies of mutant PDGF beta receptors in PC12 cells demonstrated that elimination of PI3K, phospholipase Cγ, Gap, and Syk activation did not block PDGF up-regulation of c-fos (56). In A431 cells, activation of both wild type and mutant PDGF beta receptors that associate preferentially with phospholipase Cγ increased c-fos expression, however, a mutant PDGF beta receptor that engages PI3K preferentially stimulated c-fos very little (57). In studies of α adrenoreceptors in oligodendrocyte progenitors, the MEK inhibitor PD98059 blocked MAPK activation and norepinephrine-induced increase in c-fos expression (58). Interestingly, in this system, the PI3K inhibitors LY294002 and wortmannin also attenuated MAPK activation, suggesting significant crosstalk between these two signaling pathways in these cells. Given this capacity for crosstalk between and among signaling pathways, it might be considered somewhat surprising that Epo’s two signals to the c-myc gene appear to be so functionally discreet and independent. We speculate that this dual regulation of c-myc gene expression, coupled with the potential for altered mRNA and Myc protein stabilities, afford the hematopoietic cell multiple levels of control over Myc levels during the various phases of cell growth and differentiation.

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