c-myc Is an Erythropoietin Early Response Gene in Normal Erythroid Cells: Evidence for a Protein Kinase C-Mediated Signal

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The proto-oncogene c-myc has been identified as an early response gene for erythropoietin (Epo) in transformed murine erythroleukemia cells. Epo activation of c-myc in these cells requires protein kinase C. We now show the fidelity of this signaling pathway in normal erythroid cells isolated from the spleens of phenylhydrazine-treated mice. Mouse spleen cells rich in erythroid progenitors were washed free of endogenous Epo and then incubated in the absence of Epo. Subsequent addition of Epo for 1 hour led to a dramatic elevation of c-myc transcript. Addition of the protein synthesis inhibitor cycloheximide did not prevent the c-myc response, thus identifying c-myc as an Epo early response gene in normal cells. We used this c-myc response as a reporter for signals initiated by the Epo receptor. Using a series of inhibitors with known specificities and established rank orders of potency for different kinases, we determined that the c-myc response to Epo was blocked with the following rank order: staurosporine > H7 > sangivamycin > H8. This sequence is identical to that obtained using transformed cells and is diagnostic of a protein kinase C-dependent signal. Because direct activation of protein kinase by phorbol esters does not induce terminal differentiation of normal cells, the pathway to c-myc established by these studies must represent one part of a signal transduction mechanism.

A DETAILED understanding of the mechanism of action of erythropoietin (Epo) on the molecular level is critical to the elucidation of the developmental biology of the red blood cell (RBC). Moreover, it is of potentially great importance in the design of new therapeutic modalities for disorders of RBC production. Previous studies have focused on demonstrating changes in potential signal transduction intermediates and/or perturbing these intermediates and measuring an effect on erythroid cell growth or differentiation.14 The results of these studies have been contradictory, in part due to the uncertainty that the intermediates measured are directly involved in the receptor-nucleus signal and to the multistep process leading to the observed changes in growth or differentiation.

We hypothesized that we could initiate a molecular dissection of the receptor-nucleus signal by identifying an early response gene, a gene rapidly upregulated by Epo and whose induction does not require new protein synthesis. Studies of the action of Epo on murine erythroleukemia cells showed that Epo increased the levels of c-myc transcript.1 Our recent studies of Epo-sensitive Rauscher murine erythroleukemia cells confirmed this and indicated that, in these transformed cells, c-myc induction by Epo was insensitive to the protein synthesis inhibitor cycloheximide; i.e., c-myc was an Epo early response gene.59

We have now investigated the expression of c-myc and the fidelity of its response to Epo in normal murine erythroid cells. Our results indicate that c-myc is an Epo early response gene in these cells. Moreover, transduction of the signal from the Epo receptor to c-myc requires the activation of protein kinase C (PKC).

MATERIALS AND METHODS

Cell preparation and induction studies. C57BL/6 mice less than 10 weeks old were injected subcutaneously on day 1 with a sterile solution of 2 mg phenylhydrazine/mL in phosphate-buffered saline (PBS) 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, pressed between two microscope slides, and the cells were suspended in cold Eagle medium, α-modification (α-MEM) (GIBCO, Grand Island, NY). Single cell suspensions were 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 mol/L Tris-HCl, pH 7.5) was added to lyse erythrocytes. After 10 minutes on ice, the cells were washed again and plated at 5 × 105 cells/mL in 37°C α-MEM with 10% fetal bovine serum (HyClone, Ogden, UT).

Mice treated with phenylhydrazine develop a brisk hemolytic anemia, and their spleens are greatly enriched with Epo-sensitive differentiating erythroid cells (PHZ cells).66 Because PHZ cells are exposed to high levels of endogenous Epo while in the spleen, we hypothesized that this circulating Epo might upregulate the level of c-myc transcript in vivo. Therefore, in preliminary experiments we incubated explanted PHZ cells in vitro for specified times in the absence of Epo and measured c-myc transcript levels. As predicted, cells preincubated for 3 to 4 hours showed a significant decrease in c-myc transcript from that found in cells that were not preincubated. We performed all subsequent experiments on cells that had been preincubated in the absence of Epo for 4 hours at 37°C, 5% CO2, to allow these relatively high levels of endogenous c-myc transcript to achieve a lower steady-state level (see Results). Then cells were incubated in the absence or presence of specified concentrations of highly purified, recombinant human Epo (specific activity = 210,000 IU/mg) (generous gift of Elanex Pharmaceuticals, Inc, Bothell, WA). In some experiments, cells were preincubated in the presence of the protein synthesis inhibitor cycloheximide (Sigma Chemical Co, St Louis, MO) or were incubated in the presence of the protein kinase inhibitors H7, H8 (Seikagaku, America, St Petersburg, FL), staurosporine (Calbiochem, San Diego, CA), or sangivamycin (National Cancer Institute, Bethesda, MD; NSC-65346).

The efficacy of cycloheximide as a protein synthesis inhibitor in these cells was verified by measuring the incorporation of [3H]leucine into trichloroacetic acid precipitable protein. Preincubation of

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cells for 1 hour in the presence of 10 μg or 20 μg cycloheximide/mL, followed by [³H]leucine labeling resulted in a 92% ± 2% or 94% ± 3% inhibition of protein synthesis, respectively, compared with cells not exposed to cycloheximide.

RNA extraction and Northern blot analyses. After incubation, the cells were washed with cold PBS and RNA was extracted following the method of Hatch and Bonner. RNA was resolved by electrophoresis in agarose/formaldehyde gels, transferred to GeneScreen Plus filters (DuPont/NEN, Boston, MA), and probed sequentially with ³²P-labeled cDNA corresponding to c-myc and to the “housekeeping” gene glyceraldehyde-3-phosphate dehydrogenase (GPDH). After exposure to autoradiographic film, the relative intensities of c-myc and GPDH transcript on a single filter were determined by quantitative horizontal scanning laser densitometry (LKB, Uppsala, Sweden). Percent inhibition of the Epo-stimulated c-myc response was calculated as follows:

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\text{Inhibition, } \% = \left( \frac{\text{I.A.U.}_{\text{Epo}} - \text{I.A.U.}_{\text{CHX}}}{\text{I.A.U.}_{\text{Epo}} - \text{I.A.U.}_{\text{off}}} \right) \times 100
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where I.A.U. = integrated absorbance units of c-myc determined by scanning densitometry; +Epo = in presence of Epo; −Epo = in absence of Epo; +I = in presence of inhibitor; −I = in absence of inhibitor.

Replicate scans resulted in ±3% variability. Therefore, results were rounded off to the nearest 10%. (see Figs 3 through 5).

[³H]thymidine incorporation. The effect of specified concentrations of Epo on DNA synthesis in PHZ cells was demonstrated using the [³H]thymidine incorporation assay developed by Krystal.

RESULTS

Epo treatment of PHZ cells in vitro caused a marked increase in c-myc transcript. c-myc transcript was increased by an average of fivefold after 60 minutes of treatment (mean of 12 experiments) (Fig 1). After 3 hours of Epo treatment, c-myc was increased an average of threefold over controls (mean of 5 determinations. All SD are <5% of the mean. See Materials and Methods and Results.

C-myc mRNA was superinduced by Epo in the presence of cycloheximide (Fig 1), a phenomenon characteristic of immediate/early response genes observed in other systems.

A more general measure of the Epo-responsiveness of PHZ cells is provided by measurement of [³H]thymidine incorporation into newly synthesized DNA of PHZ cells. Points represent the means of triplicate determinations. All SD are ±5% of the mean. See Materials and Methods and Results.

Our previous studies had demonstrated a PKC requirement for Epo induction of c-myc in Rauscher virus-transformed murine erythroleukemia cells. However, other evidence indicates that gp55, the membrane glycoprotein encoded by the env gene of Friend spleen focus-forming virus, can bind to the Epo receptor resulting in growth-factor independent cell proliferation. A similar action of the homologous protein of Moloney leukemia virus has been reported. In view of the homology of the Rauscher virion envelop glycoprotein, gp70, with these other two proteins, and our demonstration that Epo activates the receptor on both Friend and Rauscher cells, we considered the possibility that Epo’s signaling pathway in normal (nontransformed) erythroid cells might differ from that described in Rauscher cells. Therefore, to assess the fidelity of the PKC requirement in normal erythroid cells (PHZ cells), we measured the c-myc response in the presence of protein kinase inhibitors of different classes with known specificities and rank-orders of potency for PKC.

We first tested two chemically related agents, H7 and H8, whose established potency toward PKC in vitro and in vivo is H7 > H8. Thus, if PKC is required for the Epo-induced
c-myc response in PHZ cells, H7 should block this induction more effectively than H8. As shown in Fig 3, H7 completely inhibited the Epo-induced c-myc response at a concentration of 10 μmol/L (Fig 3A). In contrast, H8 had no effect on the c-myc response at 30 μmol/L, inhibited 30% of the c-myc response at 60 μmol/L, and 90% at 120 μmol/L (Fig 3B). The average inhibitory effect of H7 (with number of experiments) was: 5 μmol/L = 30% (2); 10 μmol/L = 60% (3); 20 μmol/L = 100% (3). The average inhibitory effect of H8 (with number of experiments) was: 30 μmol/L = 0% (3); 60 μmol/L = 40% (3); 120 μmol/L = 90% (3). Thus, the differential sensitivity of the c-myc response to these drugs matched their established differential potency toward PKC. Interestingly, 20 μmol/L H7 reduced the c-myc level below that seen in cells not treated with exogenous Epo (Fig 3A), suggesting that the basal c-myc level is modulated by a PKC-dependent mechanism.

No significant changes were seen in GPDH transcript levels, confirming the specificity of these results.

We next examined the efficacy of three other kinase inhibitors of different chemical classes in blocking the c-myc response. The microbial alkaloid staurosporine is a very potent PKC inhibitor, effective at concentrations 100-fold lower than H7 (ie, potency of staurosporine > > H7). As shown in Fig 4A, staurosporine inhibited the c-myc response virtually completely at concentrations of 80 to 160 nmol/L, nearly 100-fold lower than H7. These results represent one of two experiments that showed a similar degree of c-myc inhibition. The nucleoside analogue sangivamycin inhibits PKC in vitro and in cultured cells with a potency somewhat lower than H7 (H7 ≥ sangivamycin). As shown in Fig 4B, sangivamycin was on average 50% less potent than was H7 (Fig 3A) at blocking the c-myc response, coinciding with its reported potency against PKC relative to H7. The average inhibitory effect of sangivamycin in three experiments was: 7.5 pmol/L = 20%; 15 pmol/L = 50%; 30 pmol/L = 90%. Therefore, the previously established rank-order of potency toward PKC of the kinase inhibitors used—staurosporine > > H7 > sangivamycin > H8—coincides with that found in our experiments, consistent with our results in transformed cells. We found no inhibition of the c-myc response by W7, an inhibitor of calcium/calmodulin-dependent kinases, at the highest concentration tested (100 μmol/L; not shown).

The inhibitors described above blocked the Epo-induced c-myc response in a concentration-dependent manner and with a rank-order of potency indicative of a specificity for PKC. To confirm that this profile of inhibition is specific for PKC in PHZ cells, we tested the effectiveness of the kinase inhibitors against a c-myc response induced by a known activator of PKC, 12-O-tetradecanoylphorbol-13-acetate (TPA). TPA treatment of PHZ cells led to an average threefold elevation of c-myc transcript levels (Fig 5) (a mean of four experiments), somewhat less than that achieved with Epo. The TPA-induced c-myc response was blocked by...
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**D I S C U S S I O N**

In the present study we have shown that PKC is a necessary component of the signaling pathway activated by the Epo-receptor interaction, which leads to increased expression of the proto-oncogene c-myc in normal erythroid cells. Moreover, we have shown that c-myc is an Epo early response gene in these cells, confirming our previous studies in transformed cells.5,9

We optimized conditions for measuring an early Epo response by depriving PHZ cells of Epo immediately after removal from the mouse. Having an easily assayable response gene downstream of the Epo receptor provided a means to study the Epo signaling pathway. The magnitude and reproducibility of the c-myc response in preincubated PHZ cells enabled us to identify a PKC requirement for Epo signaling to c-myc. We have obtained analogous results (not shown) using normal erythroid cells from thiamphenicol-treated mice.29

PKC is a serine/threonine protein kinase known to be involved in transducing signals initiated by the binding of a number of peptide hormones.30 The phosphotransferase activity of PKC is elevated by diacylglycerol. Tumor-promoting phorbol esters such as TPA bind to the diacylglycerol site and activate PKC.30 We show here that TPA, like Epo, elevates c-myc transcript levels in PHZ cells. There are several isoforms in the PKC family of genes. These PKC isoforms exhibit differences in tissue distribution and subcellular localization, as well as differences in their cofactor requirements for activation.31 As not all PKCs are equally responsive to TPA, it is not certain that TPA and Epo activate the same PKC. For example, PKC-\(\beta\) in rat leukemia cells responds more rapidly to TPA than does PKC-\(\alpha\) in the same cells.32 In contrast, only PKC-\(\alpha\) is activated by TPA in canine kidney cells containing both \(\alpha\) and \(\beta\) isoforms of PKC.33 Also, it is not certain that TPA-sensitive isoforms are expressed at all stages of normal cell differentiation. Recent studies support a broader role for PKC in erythroid differentiation.34,35

Our studies with Rauscher virus-transformed erythroleukemia cells established a requirement for PKC in Epo signaling to c-myc. However, in contrast to our results with PHZ cells, activation of PKC by TPA was not sufficient for increasing c-myc transcript levels in Rauscher cells.3 The reason for this difference between transformed and normal erythroid cells is unclear. Erythroleukemia cells are a clonal population presumably representative of a single stage of the progenitor cell differentiation pathway. PHZ cells are 70% to 90% erythroid, but consist of a mixture of cell types. It has not been established which class(es) of PHZ cells is (are) responsive to TPA. Thus, TPA and Epo might not be acting on the precisely same cells, or might even be acting on different PKCs in the same cells. The identification of common substrates phosphorylated by Epo and TPA in PHZ cells may help to resolve this question.36

An elucidation of the complete repertoire of Epo-induced early response genes will enhance our understanding of the control of erythropoiesis. Toward this end, we performed preliminary experiments using Rauscher murine erythroleukemia cells37 to determine whether other genes associated with growth factor responses, namely, c-fos, junB, and egr-1 might be regulated by Epo. However, we detected no reproducible change in these transcript levels. Although these findings remain to be confirmed in homogeneous populations of nontransformed erythroid cells, this apparent deviation from the prototypical serum response is supported by several other findings. For example, c-fos, c-jun, and egr-1 are induced in myocardial cells by phorbol esters,38 and the increase in egr-1 seen after Ig-mediated B-cell activation requires PKC.37 In contrast, interleukin-6 induction of cell proliferation after G-1 arrest of B-cell hybridomas occurs without changes in c-fos, c-jun or egr-1.39 In addition, the upregulation of egr-1 in macrophages by granulocyte-macrophage colony-stimulating factor does not require PKC.40 Because the promoter of junB (and, possibly, other early response genes) is activated by several stimuli, eg, serum, phorbol esters and activated PKA,41 the specificity for early response gene activation and, indeed, the decision to activate or inhibit must reside upstream in the network of biochemical reactions that constitute the signaling pathway. This may be expected to differ among cell types, even in response to the same initial stimulus. Clarification of this difficult problem will require further studies of the early response gene products and their functions during erythroid differentiation.31

A C K N O W L E D G M E N T

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REFERENCES


27. Loomis CR, Bell RM: Sangivamycin, a nucleoside analogue, is a potent inhibitor of protein kinase C. J Biol Chem 263:1682, 1988


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