Changes in redox affect the activity of erythropoietin RNA binding protein

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Abstract We have previously identified a cytosolic protein, erythropoietin RNA binding protein (ERBP), which is up-regulated in certain tissues in response to hypoxia. To further characterize the interaction of ERBP and erythropoietin (EPO) mRNA, we have examined the role of reduction–oxidation in the EPO mRNA binding mechanism of ERBP isolated from human hepatoma cells (Hep3B). Reducing agents dithiothreitol (DTT) and 2-mercaptoethanol (2-ME) increased ERBP binding activity in a concentration-dependent manner, whereas the oxidizing agent, diamide, abolished ERBP binding activity. In addition, treatment of Hep3B cell lysates with the irreversible sulfhydryl alkylating agent *N*-ethylmaleimide resulted in inhibition of the EPO mRNA–ERBP complex. Taken together, these findings suggest that sulfhydryl groups may play a role in vivo in the regulation of EPO production through the modulation of ERBP binding activity.

Key words: Erythropoietin; Redox; RNA binding protein

1. Introduction

Post-transcriptional regulation of EPO production has been observed [1], and the regulatory mechanisms for control at this level of gene expression are currently being investigated. The modulation of mRNA turnover rates is an important mechanism of gene control which may directly influence cytokine/growth factor secretion by altering the amount of translatable RNA [2]. Specific *cis* elements have been identified that directly affect mRNA lability, while *trans* factors have been shown to bind to both 5′ and 3′ untranslated regions (UTR) of target mRNAs. Examples of these types of factors include AUBF, which stabilizes AUUUA sequences in GM-CSF; interferon-γ; interleukin-3; c-*fos*; and v-*myc* mRNAs [3–5], and IRE-BP that binds to iron responsive elements in the 5′-UTR of ferritin mRNA and the 3′-UTR of transferrin receptor mRNA [6]. We have previously identified a cytosolic protein that binds specifically to the 3′-UTR of EPO mRNA [7] and is up-regulated by hypoxia in certain tissues. Although this protein, called ERBP, is proposed to affect the stability of EPO mRNA, its specific roles have not been elucidated. Because both AUBF and IRE-BP have been shown to be affected by reduction–oxidation [5,8], we examined the possibility that ERBP was similarly regulated by changes in redox.

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Abbreviations: EPO, erythropoietin; ERBP, erythropoietin RNA binding protein; DTT, dithiothreitol; 2-ME, 2-mercaptoethanol; AUBF, adenosine-uridine binding factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IRE-BP, iron-responsive element-binding protein; 3′-UTR, 3′-untranslated region; MEM, minimal essential medium; FBS, fetal bovine serum; NEM, *N*-ethylmaleimide; PBS, phosphate buffered saline.

2. Materials and methods

2.1. Cell culture

Hep3B cells obtained from ATCC (Rockville, MD) were routinely cultured in Eagle's MEM supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin G, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2, 95% air at 37°C. Media and all supplements were purchased from Life Technologies Inc. (Gaithersburg, MD). Hypoxic conditions were obtained by placing cells in a hypoxic chamber (Billups-Rothenburg, Del Mar, CA) (1% O_2, 5% CO_2, 94% N_2) for 8 h.

2.2. Preparation of lysates

After trypsinization (0.05% trypsin and 0.53 mM EDTA; Gibco), Hep3B cells were pelleted at 800 rpm and washed twice with PBS (without calcium and magnesium). Following the second wash, the cells were gently resuspended, transferred to an Eppendorf tube, pelleted at $1000 \times g$ for 5–10 s, PBS removed and 25 mM Tris (pH 7.9), 0.5 mM EDTA added so that the final concentration was about 2.0×10^5 cells/μl. Cells were frozen and thawed through repetitive cycles [7]. The protein concentration of each cytoplasmic lysate was determined using the micro BCA (Pierce, Rockford, IL) or Bradford (Bio-Rad, Irving, CA) methods, as described by the manufacturer.

2.3. In vitro transcription

EPO cDNA was kindly provided by Dr. Jerry Powell (University of California, Davis, CA) and subcloned into the *Eco*RI site of pcDNAI (Invitrogen, San Diego, CA). Radiolabeled EPO mRNA was produced by T7 RNA polymerase (Promega) from 1 μg *Xho*I-digested pcDNAI containing EPO cDNA at the *Eco*RI site and labeled with [^{32}P]UTP [7]. Following phenol/chloroform extraction, transcription mixtures were passed through push-trap columns (Stratagene, La Jolla, CA). Specific activity was typically 10^7 cpm/μg mRNA.

2.4. Band-shift assay

2 μg of cytoplasmic lysate were incubated for 10 min with reducing agents, oxidizing agents, or vehicles followed by 5×10^4 cpm of EPO mRNA in 12 mM HEPES (pH 7.9), 10% glycerol, 15 mM KCl, 0.25 mM EDTA, 5 mM $MgCl_2$ and 200 μg/ml yeast transfer RNA in a total volume of 10 μl for 10 min at 30°C. Twenty units RNase T1 were added, and reaction mixtures were incubated for an additional 30 min at 37°C prior to electrophoresis in a 7% native polyacrylamide gel with 0.25 × TBE (Tris-borate-EDTA) running buffer. After drying, gels were exposed overnight to film (Amersham-Hyperfilm) with two intensifying screens. For quantitation of resulting gel band-shift bands the dried gel was exposed to a phosphorimaging board (Fuji Photo Co., Japan) for 30 min to 1 h, and the results were analyzed using MacBAS analyzing software.

3. Results

3.1. Comparison of normoxic and hypoxic lysates treated with various concentrations of DTT and 2-ME

It has been hypothesized that ERBP plays a role in EPO mRNA activity [7] and that it may mediate ribonuclease activity

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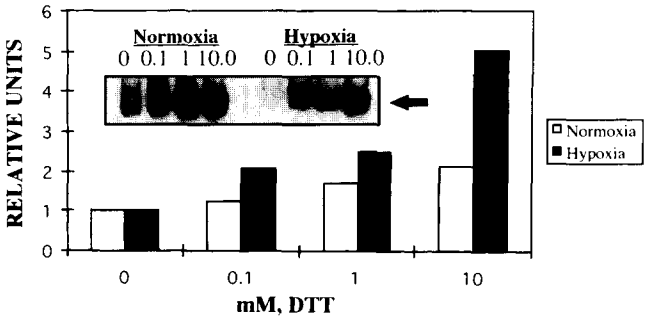

Fig. 1. Effects of dithiothreitol. Hep3B lysates were prepared from cells harvested after 8 h normoxic or hypoxic stimulus as described in section 2. Subsequently, these lysates were pretreated for 10 min with varying concentrations of DTT (0–10 mM) prior to gel band-shift assay and separation on 7% native polyacrylamide gel as described in section 2. Radioactive values for each band were obtained after exposing the dried gels to a Fuji phosphorimaging board. The data presented are the means of duplicate samples performed with lysates from three independent experiments. Standard error values for each sample are: normoxia, 0.1 mM (±0.133), 1 mM (±0.37), 10 mM (±0.82); hypoxia, 0.1 mM (±0.59), 1.0 mM (±0.34), and 10 mM (±1.38). The insert shown is a representative autoradiogram used in data analysis.

[1]. However, it is not known how the binding activity of ERBP is regulated, or if other factors recruited or bound to ERBP regulate its activity. In addition, it is known that the EPO mRNA concentration reaches a peak level after 5–8 h of hypoxia [1], suggesting that the effect of ERBP on EPO mRNA should occur at this time. In order to investigate the redox status of ERBP, normoxic and hypoxic (8 h) lysates were treated with various concentrations of DTT (0–10 mM) and 2-ME (0–10%), and their ERBP binding activity was assessed by band-shift assay. Fig. 1 shows that there is a dose-dependent increase in EPO mRNA binding by ERBP as a result of increasing amounts of DTT. In addition, this effect is observed in both normoxic and hypoxic treated lysates. Similarly, treatment with increasing amounts of 2-ME resulted in increasing ERBP binding activity (Fig. 2). At 10% 2-ME treatment, a smear attributed to denaturing of the whole cell lysate was consistently observed. These results suggest that ERBP–EPO mRNA binding might involve prior, local reduction of ERBP's sulfhydryl groups.

3.2. *Effect of reducing and oxidizing agents on the formation of ERBP–EPO mRNA complex*

To determine further the effect of reducing and oxidizing agents on the formation of ERBP–EPO mRNA complex, 2 μg of normoxic Hep3B lysates were pre-incubated for 10 min at 30°C with 0.2 mM DTT, 1% 2-ME or the oxidizing agent diamide (1 or 5 mM), with or without 1% 2-ME prior to the addition of radiolabeled EPO mRNA. This treatment was followed by RNase T1 digestion, electrophoresis and autoradiography. As above, treatment of lysates with DTT or 2-ME resulted in an increase in ERBP binding activity whereas treatment with diamide abolished ERBP binding activity. Binding activity after diamide treatment could be restored by a brief incubation with 0.1% 2-ME. A representative band-shift assay is shown in Fig. 3. The observed slower migrating band has been shown previously [7] to be competed out with GM-CSF mRNA transcripts, and its relationship to EPO mRNA binding is still being investigated. These data indicate that alteration in the redox state of the cytoplasmic lysate affects ERBP binding activity.

3.3. *Effect of N-ethylmaleimide (NEM), an irreversible oxidizing agent, on the formation of ERBP–EPO mRNA complex*

Reduced sulfhydryl groups can function in different ways. We have previously demonstrated that ERBP is composed of two subunits, 70 and 130–140 kDa, that may be attached via intermolecular disulfide bonds [7]. Likewise, AUBF is composed of three subunits, and redox changes appear to affect its intermolecular disulfide bonds [5], while redox changes appear to affect sulfhydryl groups on IRE-BP that mediate ligand binding [8]. In order to determine which of these possible interactions is operative for ERBP–EPO mRNA complex formation, Hep3B lysates were treated with NEM prior to band-shift assay. NEM differs from diamide by irreversibly alkylating reduced sulfhydryl groups. In addition, NEM has no effect on -SH groups of mRNA binding proteins involved in transient Michael adduct formation with ligands [8]. As shown in Fig. 4, pre-incubation of lysates with NEM completely abolished ERBP binding activity. NEM added after ERBP–EPO mRNA complexes were formed did not inhibit binding, although it seems to have decreased the binding activity. The effects observed after combination treatment with 2-ME are not as straightforward and may reflect partial activation of free, unalkylated sulfhydryl groups within ERBP. These observations suggest that the -SH groups of ERBP might be involved in the ERBP binding site as they appear to be for IRE-BP. Additionally, there might be -SH groups, the redox state of which might affect complex formation, residing elsewhere on the protein.

4. Discussion

In order to better understand the functions of ERBP in EPO

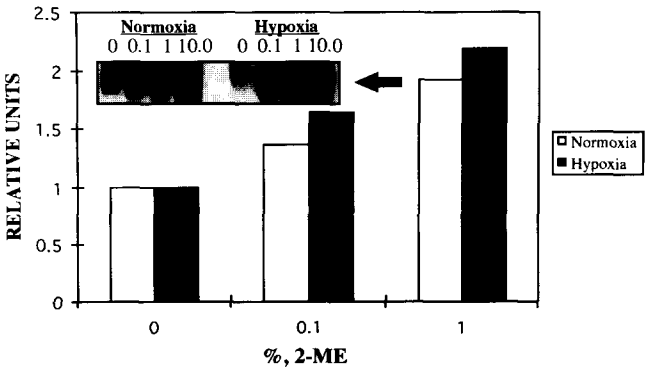

Fig. 2. Effects of 2-mercaptoethanol. Comparison of normoxic and hypoxic (8 h) lysates treated with various concentrations of 2-ME. Reaction mixtures were as described, except for the pretreatment of the lysates with different concentrations of 2-ME for 10 min prior to the addition of the radiolabeled EPO mRNA. The standard error values for each sample are: normoxia, 0.1 mM (±0.19), 1.0 mM (±0.24); hypoxia, 0.1 mM (±0.42), and 1.0 mM (±0.67). (Consistently, 10 mM 2-ME treatment of the lysates resulted in a retention of large protein complexes in the native gel. For this reason, this particular sample has been omitted from the densitometric data graph.) The arrow indicates ERBP–EPO mRNA complex.

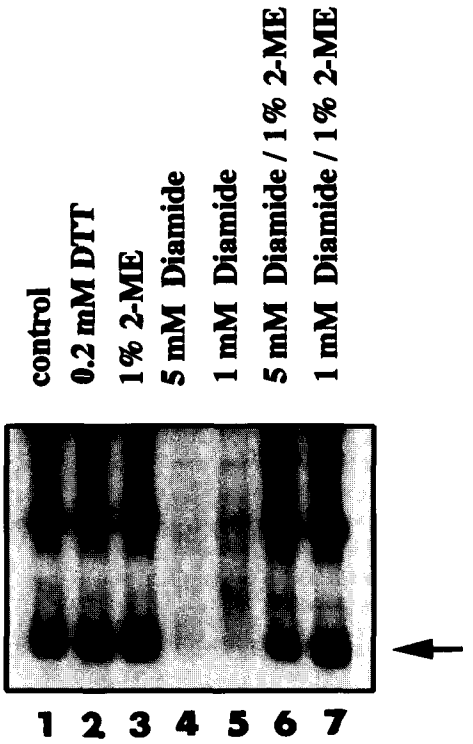

Fig. 3. Effect of reducing and oxidizing agents on the formation of ERBP–EPO mRNA complex. Normoxic Hep3B cytoplasmic lysates from near confluent cultures were prepared by freeze–thaw lysis as described in section 2. mRNA was transcribed by T7 RNA polymerase and labeled with [^{32}P]UTP from *Xho*I-digested pcDNAI containing EPO cDNA at the *Eco*RI sites. 2 μg of cytoplasmic lysate were incubated for 10 min with reducing agents, oxidizing agents or vehicle followed by 5×10^4 cpm of mRNA in 12 mM HEPES and 200 μg/ml *E. coli* transfer mRNA in a total volume of 10 μl for 10 min at 30°C. Twenty units mRNase T1 were added, and reaction mixtures were incubated for an additional 30 min at 37°C prior to electrophoresis in a 7% native polyacrylamide gel with 0.25 × TBE running buffer. After drying, the gel was exposed overnight to film. Lane 1, vehicle/control; lane 2, 0.2 mM DTT; lane 3, 1% 2-ME; lane 4, 5 mM diamide; lane 5, 1 mM diamide; lane 6,7, 5 and 1 mM diamide, respectively, followed by an additional 10 min incubation with 1% 1-ME. The arrow indicates ERBP–EPO mRNA complex.

mRNA stability, we examined the regulation of this protein in response to changes in reduction–oxidation conditions, which are known to affect other mRNA binding proteins. ERBP binding activity is sensitive to changes in redox since reducing agents increase binding activity and oxidizing agents abolish it. We conclude that free sulfhydryl groups are required for ERBP–EPO mRNA complex formation. These -SH groups most likely form transient Michael adducts with mRNA, since NEM does not affect an already formed ERBP–EPO mRNA complex. This observation is consistent with those reported for other mRNA binding proteins, such as the coat protein of *R17* coliphage [9] and the phage replicase mRNA between the *E. coli* Ala-t-RNA synthetase and its target RNA [10], and between the mammalian IRE-BP and the IRE [8]. Treatment of both normoxic and hypoxic (8 h) lysates with reducing agents resulted in a concentration-dependent increase in complex formation. However, over the course of the experiments there seemed to be a slightly greater magnitude of activatable ERBP in treated hypoxic lysates. It is possible that the hypoxic treatment results in the production of greater amounts of reduced ERBP. Interestingly,the differences seen with 2-ME treatment of normoxic lysates compared with hypoxic lysates were observed only after 5–8 h of hypoxic treatment; it was not seen at later times (20 h). The 5–8 h range coincides with the period reported to have the highest amount of EPO mRNA [1]. These observations strongly suggest that hypoxia affects the binding of ERBP by changes in redox. Previous studies from our laboratory have shown that reactive oxygen metabolites cause an increase in EPO production in renal carcinoma cells [11]. These reactive oxygen metabolites may be generated during discontinuous hypoxia, a relatively more effective means of generating EPO than is continuous hypoxia. McCord [12] has demonstrated that reactive oxygen metabolites accumulate following reperfusion and reoxygenation of blood flow in the kidney. It is also possible that the waxing and waning of flow could provide for the formation of reactive oxygen metabolites. In addition, it is believed that two major factors determine the redox state of sulfhydryl groups within the cytosol. One is the ratio of reduced-to-oxidized glutathione [13], and the second is the oxidation equilibrium constant for a particular sulfhydryl group within a protein. The equilibrium constant can vary over many orders of magnitude, reflecting the effects of a local environment around the cysteinyl moiety on its k_{ox} [14]. Conformational changes, such as interactions with the heat shock proteins (e.g. hsp70), can alter the local environment of the cysteinyl moiety and, therefore, affect the k_{ox} of a particular -SH group.

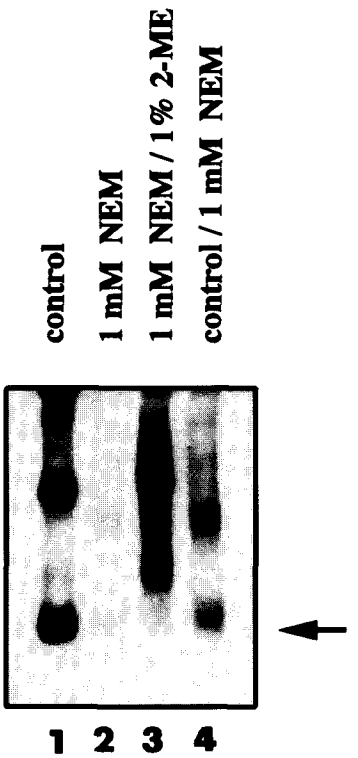

Fig. 4. Effect of *N*-ethylmaleimide, an irreversible oxidizing agent, on the formation of ERBP–EPO mRNA complex. Reaction mixtures were as described in Fig. 2, except for the pretreatment of the lysates. Lane 1, vehicle; lane 2, 1 mM NEM; lane 3, 1 mM NEM followed by 10 min incubation with 1% 2-ME; lane 4, treatment of the mixture with 1 mM NEM after the complex has already formed. The arrow indicates ERBP–EPO mRNA complex.

ERBP regulation thus far appears to be very similar to that of AUBF and IRE-BP. That is, the activity of these proteins is not related to a change in their synthesis but rather to post-translational modifications, such as phosphorylation for AUBF [5] and sensitivity to iron [15] and hemin [16], as is the case for IRE-BP. The mechanisms of ERBP regulation are undoubtedly as complex as they are for AUBF and IRE-BP. In this study, we demonstrate that in vitro detection of ERBP binding activity is dependent on a reducing environment. Conversely, binding activity can be abolished by the reversible oxidizing agent, diamide, and reconstituted by DTT or 2-ME. While these experiments were conducted on crude lysates and thus must be cautiously interpreted, the data suggest that alterations of ERBP's redox state may participate in the regulation of the protein's binding activity. Details on the mechanisms for its regulation await purification of ERBP. Although the effect of ERBP on EPO mRNA half-life has not yet been determined, we propose that the binding of ERBP to EPO mRNA subsequently targets EPO mRNA for degradation and, thus, EPO production is limited; whereas, during hypoxia, sulfhydryl groups on ERBP are not accessible for ERBP to interact with EPO mRNA and, as a result, EPO mRNA accumulates, resulting in an increase in EPO production.

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References

[1] Goldberg, M.A., Gaut, C.C. and Bunn, H.F. (1991) Blood 77, 271–277.
[2] Belasco, J. and Brawerman, G. (1993) Control of Messenger RNA Stability, Academic, New York.
[3] Malter, J.S. (1989) Science 246, 664–666.
[4] Gillis, P. and Malter, J.S. (1991) J. Biol. Chem. 266, 3172–3177.
[5] Malter, J.S. and Yin, H. (1991) J. Biol. Chem. 266, 3167–3171.
[6] Klausner, R.D., Rouault, T.A. and Harford, J.B. (1993) Cell 72, 19–28.
[7] Rondon, I.J., MacMillan, L.A., Beckman, B.S., Goldberg, M.A., Schneider, T., Bunn, H.F. and Malter, J.S. (1991) J. Biol. Chem. 266, 16594–16598.
[8] Hentze, M.W., Rouault, T.A., Harford, J.B. and Klausner, R.D. (1989) Science 244, 357–359.
[9] Starzyk, R.M., Koontz, S.W. and Schimmel, P. (1982) Nature 2908, 136–140.
[10] Romaniuk, P.J. and Uhlenbeck, O.C. (1985) Biochemistry 24, 4239–4244.
[11] Ueno, M., Brookins, J., Beckman, B.S. and Fisher, J.W. (1988) Biochem. Biophys. Res. Commun. 154, 773–780.
[12] McCord, J.M. (1985) N. Eng. J. Med. 312, 159–163.
[13] Cappel, R.E. and Gilbert, H.F. (1988) J. Biol. Chem. 263, 12204–12212
[14] Walters, D.W. and Gilbert, H.F. (1986) J. Biol. Chem. 261, 13135–13143
[15] Rouault, T.A., Hentze, M.W., Caughman, S.W., Harford, J.B. and Klausner, R.D. (1988) Science 241, 1207–1210.