Transferrin Receptor Induction by Hypoxia

HIF-1-MEDIATED TRANSCRIPTIONAL ACTIVATION AND CELL-SPECIFIC POST-TRANSCRIPTIONAL REGULATION

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The tight relationship between oxygen and iron prompted us to investigate whether the expression of transferrin receptor (TfR), which mediates cellular iron uptake, is regulated by hypoxia. In Hep3B human hepatoma cells incubated in 1% O2 or treated with CoCl2, which mimics hypoxia, we detected a 3-fold increase of TfR mRNA despite a decrease of iron regulatory proteins activity. Increased expression resulted from a 4-fold stimulation of the nuclear transcription rate of the TfR gene by both hypoxia and CoCl2. A role for hypoxia-inducible factor (HIF-1), which activates transcription by binding to hypoxia-responsive elements in the activation of TfR, stems from the following observations. (a) Hypoxia and CoCl2-dependent expression of luciferase reporter gene in transiently transfected Hep3B cells was mediated by a fragment of the human TfR promoter containing a putative hypoxia-responsive element sequence, (b) mutation of this sequence prevented hypoxic stimulation of luciferase activity, (c) binding to this sequence of HIF-1α, identified by competition experiments and supershift assays, was induced in Hep3B cells by hypoxia and CoCl2. In erythroid K562 cells, the same treatments did not affect iron regulatory proteins activity, thus resulting in a stimulation of TfR gene expression higher than in hepatoma cells.

Iron is needed for several essential functions but is also potentially dangerous as a catalyst of reactive oxygen species production (1). Thus, iron is usually bound to proteins, and iron homeostasis is tightly regulated. Iron in serum is mainly transported by transferrin (Tf),1 which delivers the metal to cells requiring it. Iron-laden Tf interacts with transferrin receptor (TfR), and the complex is then internalized by receptor-mediated endocytosis. Since the TfR plays a crucial role in the control of iron entry into cells, its expression is tightly regulated. Iron controls TfR mRNA levels through a well-characterized post-transcriptional mechanism involving binding of cytosolic iron regulatory proteins (IRPs) to iron-responsive elements (IREs) in the 3′-untranslated region of TfR mRNA (2, 3). In conditions of iron depletion, IRP actively binds to IREs and increases TfR mRNA stability by preventing access to ribonucleases (2, 3). Since, at the same time, the IRE-IRP interaction prevents ferritin mRNA translation, the combined effect of this regulation results in increased iron availability in the intracellular pool. An inverse regulation occurs when iron is plentiful in such a way that iron homeostasis is maintained. On the other hand, iron-independent TfR expression is mainly controlled at the transcriptional level (4). Indeed, elevated transcription is an important means to provide high TfR expression in erythroid (5) and mitogen-activated (6, 7) cells.

Hypoxia is increasingly recognized as an important regulator of gene expression, and a number of physiologically relevant genes have been found to be induced by hypoxic conditions that may help the cell to adapt to reduced oxygen supply (8–11). Although post-transcriptional mechanisms may contribute to the induction of hypoxia-sensitive genes, hypoxia-inducible factor (HIF-1), which activates gene transcription in response to reduced oxygen concentration, is the most relevant component of the molecular response to hypoxia. HIF-1 is a heterodimer consisting of an α and β subunit; the latter is identical to the aryl hydrocarbon receptor nuclear translocator (ARNT), which is also able to dimerize with the aryl hydrocarbon receptor. Hypoxia results in the stabilization of HIF-1α, enabling it upon dimerization with the constitutively expressed ARNT and formation of a complex with the transcriptional coactivator p300/CAF1, to bind hypoxia-responsive elements (HREs) in responsive genes.

Hypoxia stimulates erythropoiesis and intestinal iron absorption (12), and it is therefore conceivable that proteins involved in iron transport and uptake are regulated by oxygen supply. Indeed, serum Tf levels increase in animals exposed to hypoxia (13), and it has been recently shown that Tf gene expression is induced by hypoxia in hepatoma cells (14). However, for efficient erythropoiesis, transferrin must be iron-loaded and internalized through interaction of Tf with TfR, and hence, the increased plasma iron transport capacity provided by hypoxic up-regulation of Tf expression (14) should be followed by increased availability of cellular Tf binding sites.

Here we show that TfR gene expression is stimulated by hypoxia and that a HIF-1 binding site in the TfR promoter is involved in this response. Cell-specific modulation of IRP activity, by allegedly regulating TfR mRNA stability, possibly represents an additional level of control.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—Hep3B hepatoma cells were grown in minimal essential medium and K562 human erythroleukemia cells in RPMI 1640. Media were supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin. Cell lines were maintained in a humidified incubator at 37 °C in 5% CO2, 95% air. For hypoxic stimulation, cells at 90%
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RESULTS

Analysis of TfR mRNA Levels—TfR expression in response to hypoxia was studied in a line of hepatoma cells that is extensively used to investigate regulation of genes associated to hypoxic stress. Northern blot analysis (Fig. 1) showed that incubation of Hep3B cells in reduced oxygen atmosphere (1% O2) for 20 h strongly increased TfR mRNA steady state levels. Quantification of four experiments showed a 3-fold induction. Treatment with CoCl2, a well known inducer of several hypoxia-responsive genes (8–11), also increased TfR mRNA expression to a similar extent.

Bandshift Assay of IRP Activity—Since TfR is known to be regulated at post-transcriptional level by the IRE-IRP interaction (2, 3), we investigated the IRE binding activity of IRP by RNA bandshift assays in cytosolic extracts of Hep3B cells. Fig. 2 demonstrates that IRP activity in human hepatoma cells was up-regulated by iron chelation, as expected on the basis of previous work (2, 3), and markedly repressed (70% inactivation, as revealed by quantification of three separate experiments) by both hypoxic exposure and treatment with CoCl2 for 20 h, as previously shown for rat hepatoma cells (27). Treatment of cell extracts with 2-mercaptoethanol, which fully activates latent IRP (2, 3), eliminated all the differences, thus indicating equal loading of all samples and suggesting that inactivation was due to a post-translational switch. Experiments with murine cells in which, at a difference from human cells, separation and detection of IRP-1 versus IRP-2 by bandshift assay was possible, demonstrated that the two IRPs are divergently regulated by hypoxia (27, 28). We cannot specify the role of the two IRP isoforms in hypoxic human hepatoma cells; nevertheless, as IRP-1 and IRP-2 bind IREs with similar affinity and specificity (2, 3, 29), a decrease of TfR mRNA stability should reflect the observed reduction of total IRP binding activity.

Run-on Transcription Analysis—The increase of TfR mRNA levels in the presence of reduced IRF activity pointed to a transcriptional effect on TfR expression. To directly assess this aspect, we measured TfR gene transcription in isolated nuclei. Fig. 3 shows that the transcription rate of TfR gene is stimulated by both hypoxia and CoCl2, with a response similar to that of the heme oxygenase gene, which has been shown to be transcriptionally activated by hypoxia (30). On the other hand, transcription of the gene for ferritin L subunit was not altered by these treatments. Quantification of three separate experiments showed a 4-fold induction of TfR gene transcription.
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A 455-bp fragment (pTIRB-luc) was used for transfection and reporter gene assays, and its activity was compared with that of a similar construct (pTIRBm-luc) in which the HRE sequence on the sense strand had been mutated (Fig. 4A). This shorter fragment conserved high basal transcription efficiency and was still able to confer hypoxic inducibility of luciferase activity to an extent similar to that previously observed with the longer construct (Fig. 4B). In agreement with the observation that the HIF-1/HRE interaction is important for basal transcription levels of hypoxia-inducible genes also under normoxic conditions (31), the mutation somewhat decreased the basal levels of pTIRBm-luc reporter activity (data not shown), but importantly, also prevented almost completely the hypoxia-stimulated increase of luciferase activity (Fig. 4B), thus suggesting that this sequence is a functional HRE in the response of TfR to hypoxia.

DNA Binding Activity to the TfR HRE—To assess whether the sequence found in the TfR gene promoter with high homology to consensus HRE was the target of HIF-1, nuclear extracts were analyzed by electrophoretic mobility shift assays.Fig. 5, panel A shows that the TfR-18 probe detected a constitutively expressed DNA binding activity (C) as well as a DNA binding activity (HIF-1) present in hypoxic and CoCl2-treated cells (lanes 2 and 3) and absent in nonhypoxic cells (lane 1). The specificity of the interaction between the probe and hypoxia-induced factors was tested by competition with nonradioactive oligonucleotides. Inclusion of increasing amounts of unlabeled TfR-18 oligonucleotides (lanes 4–6) inhibited the binding of the constitutive and inducible complexes. Competition with cold oligonucleotides corresponding to the HRE present in the erythropoietin enhancer (W-18, lanes 7–9) suggested that the hypoxia-inducible factor binding the TfR probe was indeed HIF-1. To further determine the composition of the hypoxia and CoCl2-induced complexes, nuclear extracts were incubated with a monoclonal antibody to HIF-1α before the mobility shift assay. Supershift assays (Fig. 5, panel B, lanes 3 and 4) showed that HIF-1α interacts with the HRE sequence of the TfR gene.

TfR Gene Expression in Erythroid Cells—Erythroid cells rely almost completely on transferrin-bound iron for hemoglobin synthesis and therefore express TfR at high levels (5). Thus, to investigate whether hypoxic stimulation of TfR expression was extended to erythroid cells, we subjected K562 cells to hypoxic and CoCl2 treatment. Northern blot analysis demonstrated a marked up-regulation of the TfR transcript not only in desferrioxamine-treated K562 cells, as expected, but also in hypoxic and CoCl2-treated cells (Fig. 6, panel A). Indeed, quantification of three different experiments showed that TfR gene expression was stimulated 8-fold in hypoxic versus normoxic cells, i.e., to a greater extent than in hepatoma cells (see Fig. 1). Interestingly, DNA bandshift assays (Fig. 6, panel B) demonstrated that in erythroid cells, IRP activity was stimulated by desferrioxamine but not affected by hypoxia. These findings suggest therefore that in these cells the increased transcription of TfR gene was not counteracted by the decreased mRNA stability, which should be the result of down-regulation of IRP activity, thus resulting in higher accumulation of TfR mRNA.

Discussion

Results reported in the present paper add TfR to the growing list of hypoxia-inducible genes and thus strengthen the link between iron metabolism and oxygen homeostasis. In fact, all the major genes of iron metabolism respond to hypoxia. Although IRP-1 and IRP-2 modulation under hypoxic conditions is determined at a post-translational level (27, 28), and ferritin induction is post-transcriptionally controlled as a result of IRP inactivation (32, 33), the expression of TfR (14) and TfR (present study) is transcriptionally regulated. It is evident that there is...
post-transcriptional regulation of TfR expression via IRP-mediated control of mRNA stability in response to iron (2, 3) and other stimuli (34, 35); however, we provide here results from several lines of investigation to show that hypoxic stimulation of TfR expression is mainly transcriptional. Indeed, run-on experiments demonstrated elevated transcription of the TfR gene in hypoxic and CoCl₂-treated cells, which resulted in a rise of steady-state mRNA levels. Transfection experiments provided further evidence for the critical role of transcriptional control in the response of TfR expression to reduced oxygen concentration. We also demonstrated that hypoxia stimulates TfR gene transcription through HIF-1α, the best characterized transcriptional activator of hypoxia-sensitive genes. Indeed, a
well conserved HRE sequence is present in the human TFR promoter; moreover, when cells were transfected with constructs in which the putative HRE was mutated to abolish HIF-1 binding, inducibility by hypoxia was lost. Results of supershift assays are also consistent with HIF-1 acting as a transactivating factor in the response of TFR to hypoxia. Indication that the TFR HRE is necessary to confer transcription activation in response to hypoxia and that TFR possesses the main properties shared by HIF-1-regulated genes (11) is provided by two other types of evidence. (i) The extent of response was similar when luciferase expression was driven by both the 1.7-kilobase- and the 455-bp-long promoters, thus indicating that the latter contained the main regulatory element(s), and (ii) mutation of the HRE around –85 almost completely abolished the response to reduced oxygen concentration, thus indicating the absence of other critical sites within the shorter construct. The present results suggest that in Hep3B cells, transcriptional induction was sufficient to overcome the counteracting effect of decreased IRP activity, which, on the basis of the well known effects of the IRE-IRP interaction on TFR mRNA turnover (2, 3), is expected to have decreased TFR mRNA stability.

While this study was in preparation, it was reported that the activation of IRP binding capacity induced by hypoxia in Hep3B cells resulted in higher expression of TFR and suppression of ferritin synthesis (36). We have no immediate explanation for the discrepancy between our results, which are in agreement with previous evidence of a reduced IRP-1 binding in murine hepatoma cells and macrophages (27, 28, 32), and those of Toth et al. (36). However, with regard to the post-transcriptional control imposed by IRPs, since the two IRPs are regulated oppositely by hypoxia (28) i.e. decreased IRP-1 and increased IRP-2 activity, the discrepancy in the observed total IRP activity (IRP-1 and IRP-2) may depend on the relative abundance of the two forms in a particular cell. In turn, this can be influenced by a variety of parameters, such as iron content of the medium and proliferative status of the cell (37), which could possibly account for the divergent results obtained in different laboratories using the same cell line. On the other hand, although increased total IRP activity as the result of higher IRP-2 levels is plausible in cells rich in IRP-2, e.g. 293 human kidney cells (28), a similar up-regulation is less likely in Hep3B cells, where the amount of IRP-2 is low (36).

Although hepatic cells are a convenient system to study hypoxia, the dependence of liver cells on Tf iron is limited. Indeed, hepatic TfR expression is immediately down-regulated hypoxia, the dependence of liver cells on Tf iron is limited. Indeed, hepatic TfR expression is immediately down-regulated in response to hypoxia, the dependence of liver cells on Tf iron is limited. Indeed, hepatic TfR expression is immediately down-regulated by iron overload in patients with hemochromatosis (38) who nonetheless accumulate iron from non-transferrin sources (39).

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