Identification of a Hypoxia Response Element in the Transferrin Receptor Gene*

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Expression of the transferrin receptor, which mediates iron uptake from transferrin, is negatively regulated post-transcriptionally by intracellular iron through iron-responsive elements in the 3′-untranslated region of the transferrin receptor mRNA. Transcriptional mechanisms are also involved in receptor expression, but these are poorly understood. In this study we have characterized the transferrin receptor promoter region and identified a functional hypoxia response element that contains a binding site for hypoxia-inducible factor-1 (HIF-1). Exposure of K562 and HeLa cells to hypoxia for 16 h resulted in a 2- to 3-fold increase in transferrin receptor mRNA expression. A motif with multipartite organization similar to the hypoxia response element of a number of hypoxia-inducible genes such as erythropoietin was identified within a 100-base pair sequence upstream of the transcriptional start site. Mutation of a site similar to the consensus HIF-binding site (HBS) in this motif attenuated the hypoxic response by 80%. Transient co-expression of the two HIF-1 subunits (HIF-1α and HIF-1β) enhanced the wild type transferrin receptor promoter activity, but that which contained a mutated HBS yielded no such response. Electrophoretic mobility shift assays revealed that HIF-1 was stimulated and bound to the transferrin receptor HBS upon hypoxic challenge. Our results indicate that the transferrin receptor is a target gene for HIF-1.

The transferrin receptor is a cell membrane-associated glycoprotein that serves as a gatekeeper in regulating cellular uptake of iron from transferrin, a plasma protein that transports iron in the circulation (1, 2). Cellular iron uptake from transferrin involves the binding of transferrin to the transferrin receptor, internalization of transferrin within an endocytic vesicle by receptor-mediated endocytosis, and the release of iron from the protein by a decrease in endosomal pH (3, 4). Iron released from transferrin is then transported through the endosomal membrane, and a compelling candidate for serving this function is “natural resistance-associated macropheage protein 2” (Nramp2), also known as DCT1 (divalent cation transporter-1) (5). After its release from endosomes, iron is transported to intracellular sites of use and/or storage, and the iron-free transferrin that remains receptor-bound returns to the cell surface where it transfers iron to the cells (3, 4).

With the exception of highly differentiated cells such as erythrocytes, transferrin receptors are probably expressed on all cells, but their levels vary greatly (2, 4). Transferrin receptors are highly expressed on immature erythroid cells, placental tissue, and rapidly dividing cells, both normal and malignant. In proliferating nonerythroid cells, the expression of transferrin receptors is negatively regulated post-transcriptionally by intracellular iron through iron-responsive elements (IREs) in the 3′ untranslated region of the transferrin receptor mRNA. IREs are recognized by specific cytoplasmic proteins (iron regulatory proteins (IRPs)) that, under conditions of decreased iron in the labile pool, bind to the IREs of transferrin receptor mRNA, preventing its degradation. On the other hand, the expansion of the labile iron pool leads to a rapid degradation of transferrin receptor mRNA, which is not protected since IRPs are not bound to it (6, 7). However, some cells and tissues with specific requirements for iron probably evolved mechanisms that can override the IRE/IRP-dependent control of transferrin receptor expression. Erythroid cells, which are the most avid consumers of iron in the organism, use a transcriptional mechanism to maintain very high transferrin receptor levels (8). Transcriptional regulation is probably also involved in the receptor induction when resting cells are activated to proliferate and during T and B lymphocyte activation (2). Although the transcriptional regulation of the transferrin receptor is not fully understood, deletion analysis identified a minimal region of about 100 base pairs upstream from the transcriptional start site that drives both basal as well as serum/mitogenic stimulation of promoter activity (9, 10). This promoter region also contains elements such as the Ets-binding site and the adjacent AP-1-like sequence, necessary for the transcriptional up-regulation of the transferrin receptor during erythroid differentiation.2

Recently hypoxia was shown to increase transferrin receptor expression in a hepatoma cell line (11) and endothelial cells (12), and it has been proposed that this increase is due to the hypoxia-enhanced IRE/IRP-1 binding and consequent stabilization of the transferrin receptor message (11). However, in the

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1 The abbreviations used are: IRE, iron-responsive element; HRE, hypoxia response element; HIF-1, hypoxia-inducible factor-1; HBS, HIF-binding site; IRP, iron regulatory protein; Nramp2, natural resistance-associated macropheage protein 2; DCT1, divalent cation transporter-1; Epo, erythropoietin; VEGF, vascular endothelial growth factor; CRE, cyclic AMP-responsive element; CREB-1, CRE-binding protein; ATF-1, activating transcription factor-1; EMSA, electrophoretic mobility shift assays; TR [HBSm] and TR [CREm], reporter plasmids of the transferrin receptor promoter with mutated HBS and CRE, respectively.
2 C. N. Lok and P. Ponka, submitted for publication.
course of our studies on the transcriptional control of the transferrin receptor expression, we have noticed that the promoter region of the transferrin receptor gene contains a sequence very similar to the hypoxia response element (HRE) that mediates transcriptional activation by hypoxia-inducible factor-1 (HIF-1). HIF-1 (13, 14) is a heterodimer (HIF-1α and HIF-1β), the latter being identical to the aryl hydrocarbon nuclear translocator (AHNT) transcription factor which activates a wide range of genes encoding proteins that represent an important physiological adaptation to hypoxia (15). These genes include erythropoietin (Epo) (13, 17), vascular endothelial growth factor (VEGF) (18), several glycolytic enzymes (19), glucose transporters (19), inducible nitric-oxide synthase (20), heme oxygenase-1 (21), and transferrin (22). Hypoxia-induced activation of HIF-1 involves, at least in part, a decrease in oxygen-sensitive degradation of the HIF-1α and an increase in its binding to the HIF-1-binding site (HBS) present in the HRE (23). In this study we demonstrated that the transferrin receptor gene contains a functional HRE that binds HIF-1, which regulates receptor expression under hypoxic conditions.

EXPERIMENTAL PROCEDURES

Cell Culture—K562 cells were maintained in RPMI medium supplemented with 10% fetal bovine serum and 2 mM glutamine. For hypoxic stimulation, cells were seeded at 5 × 10⁶ cells/ml and put in modular incubator. Hypoxic conditions were produced by purging the incubator with 95% N₂/5% CO₂ at 2 pounds/square inch for 15 min, after which the incubator was sealed for 16 h. The partial pressure of O₂ of the medium in hypoxic condition was below 22 mm Hg as measured by a blood gas analyzer.

RNA Analysis—After incubation at normal or low O₂ tension, the cells were washed, and the total RNA was isolated by guanidine thiocyanate/acid phenol extraction. Total RNA (10 µg) was then added, and the incubation was continued at room temperature for a further 30 min. For supershift assays, 1 µg of anti-HIF-1α (Transduction Laboratories) or anti-Jun (Santa Cruz) antibodies was added to the complete binding reaction mixtures, and the incubation was allowed to continue for 30 min on ice. The DNA-protein complexes were resolved on 6% polyacrylamide gels as described elsewhere (13).

RESULTS

Hypoxic Regulation of Transferrin Receptor mRNA Expression—Transferrin receptor mRNA levels in cells incubated under control and hypoxic conditions were measured using Northern blot analysis. Fig. 1 shows that after the exposure of both K562 and HeLa cells to hypoxia for 16 h, the levels of transferrin receptor mRNA increase 2- to 3-fold. By contrast, decreases of β-actin mRNA were observed in cells incubated at low O₂ pressure.

HRE Is Present in the Transferrin Receptor Gene—Because the hypoxic induction of proteins often involves binding of HIF-1 to HRE in their respective genes, we examined whether a consensus HRE was present in the transferrin receptor gene. Indeed, we found that the promoter region of the transferrin receptor gene (Fig. 2) contains a sequence highly similar to the HRE found in genes for Epo (13, 17), VEGF (18), and lactate dehydrogenase A (16, 19). Most of the hypoxia response elements exhibit multipartite organization including at least one core HBS and one or two cis-acting elements that are necessary for full inducibility of the gene by hypoxia (reviewed in Ref. 24). As shown in Fig. 2, a HIF-1-binding site with the invariant sequence ROGTG is present not only in known hypoxia-responsive genes but also in the transferrin receptor gene. Seven base pairs of the putative HBS of the transferrin receptor gene (TACGTG) are identical to the HBS of several hypoxia-regulated genes such as Epo (13, 17), phosphofructose kinase L (19), inducible nitric-oxide synthase (20), and transferrin (22). Interestingly, transferrin receptor, Epo (13) and VEGF (18) genes have a CACAG sequence located in close proximity to the HBS. In addition, both the HBS in the transferrin receptor gene and the HBS in the lactate dehydrogenase A gene (16, 26) have a nearby cyclic AMP-responsive element (CRE). Fig. 2 also shows that the putative HREs in human and mouse transferrin receptor genes (sequence kindly provided by Nancy Andrews and Joanne Levy) are well conserved.

HIF-1 Binds to HBS of Transferrin Receptor Gene—To determine whether the HBS in the transferrin receptor gene is an authentic HBS that can be recognized by HIF-1, electrophoretic mobility shift assays were performed using nuclear extracts prepared from cells incubated under hypoxic and normoxic conditions and the radiolabeled oligonucleotide harboring the putative HBS of the transferrin receptor and its flanking sequence. Fig. 3 shows that this oligomer bound constitutive factors present in both normoxic and hypoxic samples. Importantly, an induced binding activity appeared in nuclear ex-
Invariant sequences are receptor genes with those of some selected hypoxia-regulated genes. The comparison of the HBS, CRE, and CACAG sequences of transferrin signed as 1. Nucleotide numbers are denoted with the transcription start site as-gene. A against the HIF-1 investigated by supershift assays using antibodies raised against hypoxia-induced factors when it was radiolabeled (Fig. 3, lane 6). As shown in Fig. 3 (lane 6), anti-HIF-1α was able to “supershift” the hypoxia-induced binding activity. Collectively these data indicate that the HBS of the transferrin receptor behaves similarly as consensus HBS and that it can bind HIF-1a. The TfR Gene Contains Functional HRE—To further investigate the function of a potential HRE of the transferrin receptor gene, a reporter gene construct linking the promoter region containing the HRE and a luciferase gene was constructed and transiently transfected into K562 cells (Fig. 4). This promoter region conferred a basal promoter activity under normoxic conditions. Exposure of the cells to hypoxia resulted in an approximately 8-fold increase in promoter activity, but disruption of the putative HBS attenuated the hypoxic stimulation of the transferrin receptor promoter activity by 80%. These data indicate that the HBS in the transferrin receptor promoter is functionally identical to the consensus HBS present in other hypoxia-regulated genes. We also tested whether the CRE site plays a role in response to hypoxia. Although a 7-fold higher activity was still observed in hypoxia-stimulated samples as compared with normoxic samples, the mutation of the CRE significantly reduced both basal and hypoxia-stimulated promoter activity (Fig. 4). Taken together, these results indicate that the CRE is specifically involved in the hypoxia-stimulated promoter activity and that the CRE site may be required for
optimal hypoxic response.

Stimulation of Transferrin Receptor Promoter Activity by HIF-1—The functionality of the transferrin receptor HRE was further studied by transient transfection of HIF-1 subunits together with the transferrin receptor HRE-luciferase reporter gene (Fig. 5). Transfections with either HIF-1α or HIF-1β subunit moderately increased the promoter activity whereas co-transfections with both α and β subunits resulted in a further significant increase in the reporter activity. In contrast, transfection of HIF-1 did not affect the activity of the luciferase reporter when the transferrin receptor HBS was mutated.

Involvement of HIF-1 in Cobalt- or Desferrioxamine-mediated Increase in Transferrin Receptor Expression—The biological response induced by hypoxia can be mimicked by treatment with cobaltous ion or iron chelators, which mediate their effects in part by activating HIF-1 (presumably through perturbation of oxygen-sensing processes) (reviewed in Ref. 15). As shown in Fig. 6, treatment of cells with either cobalt chloride or the iron chelator desferrioxamine moderately stimulated transferrin receptor-HRE reporter activity, induced HIF-1 binding to the HBS of the transferrin receptor gene in EMSA and enhanced transferrin receptor mRNA expression. These results further support the notion that the transferrin receptor gene is activated by hypoxia and that it contains functional HRE.

**FIG. 5. Stimulation of transferrin receptor promoter activity by HIF-1.** K562 cells were co-transfected with HIF-1α and β subunits, reporter plasmids of wild type transferrin receptor promoter (WT), or its HBS mutant (TR.HBSm) and a balanced amount of pGEM as described under “Experimental Procedures.” Transfection efficiencies were determined by co-transfection of SV40-β galactosidase plasmids and show <10% variation between individual transfections. The data represent relative luciferase activities (mean ± S.E., n = 3). The luciferase activities of the cells without transfected HIF-1 subunits are set at unity.

**DISCUSSION**

This study demonstrated that transferrin receptor gene is hypoxically induced in K562 and HeLa cells (Fig. 1). One possible mechanism by which hypoxia can regulate transferrin receptor mRNA expression is through the transcription factor HIF-1. Most HIF-1-regulated genes contain an enhancer sequence termed HRE, which harbors the HBS as well as one or two additional cis-acting elements in the vicinity of the HBS (reviewed in Ref. 24). Indeed, we demonstrated that the transferrin receptor promoter region contains a functional HRE in EMSA and enhanced transferrin receptor mRNA expression. These results further support the notion that the transferrin receptor gene is activated by hypoxia and that it contains functional HRE.

**Hypoxia Response Element of Transferrin Receptor Gene**

HREs of genes such as Epo and VEGF (Fig. 2) are well conserved in the corresponding mouse sequence (Fig. 2). These sequence data have, therefore, suggested that transferrin receptor HREs have functional importance. We confirmed this by electrophoretic mobility shift assays, which showed that the HBS of the human transferrin receptor gene binds HIF-1 (Fig. 3). Moreover, our studies clearly demonstrated that the wild type but not HBS-mutated transferrin receptor promoter (−118 to +14) conferred hypoxic inducibility to the luciferase reporter gene in K562 cells (Fig. 4). Furthermore, transient co-expression of the two HIF-1 subunits (HIF-1α and HIF-1β) enhanced the transferrin receptor promoter activity (Fig. 5).

These results indicate that the transferrin receptor gene contains a functional HRE. In addition, cobaltous ion, which can mimic hypoxia, also stimulated both HIF-1 binding to the HBS of the transferrin receptor and activated its promoter activity (Fig. 6). Interestingly, desferrioxamine had effects very similar to those seen with cobalt (Fig. 6). Desferrioxamine lowers intracellular iron levels and is thought to perturb a redox-based oxygen-sensing process (reviewed in Ref. 15). It is noteworthy that K562 cells treated with desferrioxamine were demonstrated to have a higher transcription rate of transferrin receptor than iron-replete cells (25). However, there is no doubt that desferrioxamine increases transferrin receptor mRNA levels via the IRE/IRP mechanisms (6, 7).

The similarity between the regulation of transferrin receptor and lactate dehydrogenase A by HIF-1 is of particular interest (Fig. 2). As already mentioned, the promoter regions of both the transferrin receptor and lactate dehydrogenase A genes contain HRE comprised of a HBS and a nearby CRE site. Although the HBS is clearly necessary for hypoxia-enhanced activity of both promoters, the CRE site is required for optimal hypoxic response. The CRE site of the lactate dehydrogenase A was shown to bind CREB-1/ATF-1 transcription factors (26). Interestingly, both HIF-1 and CREB-1/ATF-1 interact at the HRE with p300/CREB-binding protein (26, 27), the transcriptional adapter proteins that act as signaling bridges between a number of specific DNA-bound transcription factors and the basal transcription machinery. The CRE site of the transferrin receptor gene also recognizes CREB-1/ATF-1,3 and hence, it seems likely that similar molecular mechanisms are involved in the HIF-1-mediated transcription.

It is now well established that post-transcriptional regulation via IRE/IRP system plays an important role in the control of transferrin receptor expression in response to changes in intracellular iron levels (6, 7). When cellular iron becomes limiting, IRP-2 is present in the cytosol, and IRP-1 is recruited into a high affinity binding state. The binding of IRPs to the IREs in the 3′-untranslated region of transferrin receptor mRNA stabilizes this transcript. On the other hand, when intracellular iron is plentiful, IRP-1 contains a [4Fe-4S] cluster, in which form it is unable to bind to IREs, and IRP-2 is degraded. Hence, in iron-replete cells, IRPs are not available for binding to the IREs, resulting in a rapid degradation of transferrin receptor mRNA. However, iron is not the only species that modulates IRP-1 binding activity, IRP-2 levels, and consequently, transferrin receptor expression. RNA binding activity of IRP-1 can be stimulated by nitric oxide (28, 29) or hydrogen peroxide (30), suggesting that “oxidative stress” affects cellular iron metabolism.

Since cellular production of reactive oxygen species is related to O2 concentration, the effects of hypoxia on IRP-1 and IRP-2 RNA binding activity were investigated, but the results obtained are somewhat controversial. Hanson and Leibold (31)...

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3 C. N. Lok and P. Ponka, unpublished data.
showed that hypoxia inactivated IRP-1 binding to RNA and recently reported that hypoxia increased IRP-2 levels by a post-translational mechanism involving protein stability (32). However, it is unclear what the overall functional consequences of these changes are, since transferrin receptor expression after hypoxia remains to be examined. On the other hand, Toth et al. (11) found that hypoxia enhances IRE/IRP-1 binding that was associated with a significant increase in transferrin receptor mRNA levels. Our findings reported here, together with the aforementioned data (11, 31, 32), indicate that multiple hypoxia-stimulated mechanisms may exist to modulate transferrin receptor expression. It is not surprising that both transcriptional and post-transcriptional mechanisms operate to regulate transferrin receptor expression, as in the case of prototypic hypoxia-inducible genes such as erythropoietin and VEGF (reviewed in Ref. 15). Our data indicate the transferrin receptor gene possesses a functional HRE that plays a role in hypoxia-regulated transcription. It is tempting to speculate that the HRE of the transferrin receptor gene, in cooperation with other specific enhancers, is involved in the receptor transcription in a tissue- and stage-specific manner.

In addition to transferrin receptor, several other genes involved in iron transport appear to be modulated by low oxygen tension. Transferrin expression in hepatoma cells has been shown to be stimulated by hypoxia via a HIF-1/HRE-mediated mechanism (22). Moreover, a putative HBS has been identified in the promoter region of Nramp2/DCT1 (33). Taken together, these reports support a model that hypoxia may augment the overall iron transport machinery, resulting in increasing iron uptake into the cells. Although iron is involved in multiple physiological processes such as oxygen transport, oxidative energy production, and cell growth and development, the biological significance of enhanced transferrin receptor expression during hypoxia remains to be determined. The most typical physiological response elicited by hypoxia is the increase in production of erythropoietin, which stimulates erythropoiesis. Differentiation of erythroid precursors to hemoglobin-synthesizing cells is associated with an enhanced iron uptake mediated by the increase in transferrin receptor expression in the developing erythroid cells. There is no doubt that erythropoietin-mediated mechanisms directly stimulate transferrin receptor expression (8), and hence, the effect of hypoxia on receptor expression can be only partial. However, it cannot be ruled out that both erythropoietin and hypoxia can cooperate to drive maximal expression of transferrin receptors under severe hypoxic conditions. Alternatively, it will be interesting to examine the effect of hypoxia on transferrin receptor expression in heart or skeletal muscle as the enhanced synthesis of hemoproteins such as myoglobin in these tissues during hypoxia has been demonstrated (34–36).

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