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Hypoxia Induces the Expression of a 43-kDa Protein (PROXY-1) in Normal and Malignant Cells

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This study was designed to determine the expression of cellular factors that may participate in phenotypic changes that occur under conditions of hypoxia. Using the RT-PCR differential display method, we isolated a cDNA fragment corresponding to a gene whose expression was induced in trophoblast and breast carcinoma cells cultured under 1 or 2% oxygen vs 4% oxygen or higher. This gene encodes a 43-kDa protein initially identified in homocysteine-treated endothelial cells and later shown to be upregulated in various human and mouse cell types (termed RTP, Drg1, Cap43, rit42, Ndr1). Herein we refer to this gene product as PROXY-1, for Protein Regulated by OXYgen-1. Elevated mRNA and protein levels were first observed in cells cultured in 1% oxygen for 8 h. Although PROXY-1 mRNA levels returned to near-control values within 2 h of reexposure to 20% oxygen, protein levels remained high 72 h after reexposure to 20% oxygen. Treatment of cells with hypoxia mimics such as cobalt or iron chelators also increased PROXY-1 expression. Moreover, presence of 30% carbon monoxide in the hypoxic atmosphere abrogated the upregulation of PROXY-1 expression. These findings suggest that hypoxia upregulates PROXY-1 levels through a heme protein-dependent pathway and that assessment of PROXY-1 expression may be of potential use in evaluating tissue hypoxia. © 2000 Academic Press

Key Words: **hypoxia; oxygen; gene expression; protein; heme protein; mRNA; cancer.**

There is substantial evidence that local tissue oxygen levels play an important role in determining cell function and phenotype. For example, experimental data indicate that hypoxia may be causally-linked to malignant progression both by enhancing tumor cell release of angiogenic molecules such as vascular endothelial growth factor (VEGF) (1), as well as by promoting their ability to invade extracellular matrix (ECM) *in vitro* and to metastasize *in vivo* (2, 3). Similarly, our studies have demonstrated that hypoxia stimulates the *in vitro* invasive properties of both human trophoblast and breast cancer cells, an effect that is directly linked to the increased expression of the cell surface receptor for urokinase-type plasminogen activator (uPAR) (4, 5). We also determined that low levels of oxygen increase the expression of plasminogen activator inhibitor-1 (PAI-1) in immortalized human trophoblast cells and that a heme protein-based process mediates the hypoxia-induced hyperinvasiveness, as well as the uPAR and PAI-1 upregulation (4, 6). This latter mechanism is very likely equivalent to the one involved in the hypoxic upregulation of factors such as erythropoietin (7), glycolytic enzymes (8), heme oxygenase-1 (HO-1) (9), endothelin-1 (ET-1) (10–12) and tyrosine hydroxylase (13) among others. Further, hypoxia inducible factors (HIFs) alone as well as when complexed with the von Hippel-Lindau tumor suppressor protein (pVHL) have been shown to be important players in the hypoxic control of gene expression (8, 9, 14–16). However, despite the fact that significant progress has been made toward elucidating components of the mechanisms regulating cellular responses to hypoxia, it is clear that all of the regulatory elements have not been either identified or characterized. In particular, of interest are genes and gene products that are expressed in a characteristic "on–off" manner following changes in the level of oxygen. This type of expression pattern would indicate that the factor(s) plays a critical role in the adaptive response of a cell. This is in contrast to genes and gene products in which there is a more gradual response, thereby suggesting a modulatory, but not obligatory, role in the adaptive responses to low oxygen levels. The present study was designed to dis-

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cover a novel factor(s) that would typify an "on–off" role in the hypoxic regulation of cellular phenotypes. To accomplish this, we used the RT-PCR based differential display approach to demonstrate the induction of a gene in a variety of cell types cultured under very specific conditions. Based on the finding that expression of this gene undergoes a striking pattern of up-regulation under conditions of low oxygen, we propose that its product (PROXY-1) may play a role as a molecular "switch" that marks the phenotypic transition of cells to the hypoxic state.

MATERIALS AND METHODS

Cells. Human metastatic MDA-MB-231 breast cancer cells (17) and immortalized human trophoblast cells (18) were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS; Gibco BRL). Tumorigenic, but nonmetastatic, human breast carcinoma MCF10A1T3B cells (provided by Dr. Bruce Elliot, Queen's University, Kingston, ON) were maintained in DMEM/F12 supplemented with 5% FBS, 10 μg/ml insulin, 0.2 ng/ml epidermal growth factor, and 0.5 μg/ml hydrocortisone. Human aortic smooth muscle cells (HASMC), a kind gift of Dr. Keith McCrae (Case Western Reserve University, Cleveland, OH), were cultured in M199 medium (Gibco BRL) supplemented with 10% FBS.

Culture conditions. For hypoxic conditions, cells were placed in airtight chambers (BellCo Biotechnology, Vineland, NJ) which were flushed with a 5% carbon dioxide/95% nitrogen mixture until the oxygen concentration, measured with a Miniox 1 oxygen analyzer (Catalyst Research Corp., Owings Mills, MD), was 0%. Under these conditions, the oxygen concentration equilibrated within 1–2 h at approximately 1% and remained at that level throughout the entire incubation period. At these levels, the pO_2 values reached 10-15 mmHg at the bottom of the tissue culture plate as determined with a transcutaneous pO_2 analyzer (Kontron Scientific Ltd., Mississauga, ON). For culture in 2–10% oxygen, cells were incubated for 24 h at 37°C in a multi-gas (nitrogen–carbon dioxide) incubator (Forma Scientific, Marietta, OH) previously allowed to equilibrate at 2–10% oxygen, 5% carbon dioxide, balance nitrogen. Control cultures were incubated in 20% oxygen/5% carbon dioxide, for 24 h, in a Sanyo carbon dioxide incubator (Esbe Scientific, Markham, ON).

To assess whether hypoxia regulates gene expression through iron-containing heme proteins, cells were cultured for up to 72 h at 20% oxygen in the presence or absence of either 100 μM cobalt chloride, 30 mM sodium 4,5-dihydroxybenzene-1,3-disulfonate (Tiron), or 100 μM desferrioxamine mesylate (DFO) (Sigma Chemical Co., St. Louis, MO). These compounds are hypoxia mimics that have been widely used to study the role of heme proteins in hypoxic responses (4–7, 19–21). Furthermore, the potential role of a heme protein in mediating hypoxic responses was also examined by culturing cells in 1% oxygen in the presence of 30% carbon monoxide, which maintains the heme protein in the "oxy" state and thereby blocks heme protein-mediated hypoxic responses.

Cell viability was determined by measuring the levels of lactate dehydrogenase (LDH) in the culture medium of triplicate plates incubated in 20% or 1% oxygen for up to 72 h, using a kit from Sigma Diagnostics (St. Louis, MO). This assay is based on a colorimetric reaction that involves the reduction of pyruvate to lactate at a rate proportional to the amount of LDH present.

RNA extraction and mRNA differential display. Total RNA was extracted from HTR-8/SVneo and MDA-MB-231 cells cultured under either standard (20% oxygen) or hypoxic (1% oxygen) conditions by the acid guanidium phenol–chloroform. Differential display was performed according to the method of Liang and Pardee (22) using an RNAImage kit (GenHunter, Brookline, MA) as reported by us previously (23). Bands displaying differential intensities were excised, re-amplified and cloned into pCR2.1 or pCRII vectors (Invitrogen, San Diego, CA). The nucleotide sequence of the subcloned PCR fragments was determined by the dideoxynucleotide sequencing method at the Core Facility for Protein/DNA Chemistry (Queen's University, Kingston, ON). Comparison of DNA homology with the GenBank database was performed using the BLAST algorithm.

Northern blot analysis. Total RNA was isolated from cells and 20 μg aliquots were denatured, subjected to electrophoresis in 1% agarose-formaldehyde gels, transferred to nylon membranes and hybridized with [^{32}P]-dCTP-labeled cDNA probe as described previously (24). Bands on exposed x-ray film were analyzed using a SigmaGel software package (Jandel Scientific Software, San Rafael, CA). Due to the substantial effect of hypoxia on the expression of certain "housekeeping" genes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin and cyclophilin (25), 18S rRNA was used to control for differences in total RNA loading. It has been reported that rRNA levels in a variety of cell types are not affected by exposure to hypoxia (25).

Production of anti-Drg1 antiserum. After transfection of *E. coli* with an almost full-length (first two codons missing) Drg1 cDNA cloned into the pProEXHT expression vector, fusion protein was harvested from bacterial lysates by affinity chromatography with Ni-NTA agarose (Qiagen, Mississauga, ON), followed by denaturing batch purification (Qiagen). Immunization of rabbits was performed by repeated intradermal injection of 100 μg of the protein with incomplete Freund's adjuvant. Specificity of the antiserum was assessed by Western blot and immunohistochemistry. In Western blots, using protein isolated from HT29 and Caco2 colon cancer cells, as well as proteins extracted from *E. coli* cells transfected with the Drg1 construct or the empty vector, specificity was confirmed by the occurrence of a 43-kDa (Caco2 and HT29 cells) or a 53.5-kDa (recombinant) band, the latter lacking in empty vector-transfected *E. coli*.

Western blot analysis. To examine levels of PROXY-1 protein, cells were lysed with buffer containing 40 mM Hepes, pH 7.2, 100 mM NaCl, 20% glycerol, 0.1 mM EDTA pH 8.0, 0.2% Triton X-100, 1 mM DTT, and 2 mM PMSF, and centrifuged briefly at 4°C. SDS–PAGE on supernatants was performed and resolved proteins were transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). Drg1 antigen was detected by enhanced chemiluminescence (ECL, Amersham Canada Inc., Oakville, ON) using rabbit anti-Drg1 antiserum followed by peroxidase-labeled secondary antibody.

RESULTS

Identification of genes induced by hypoxia. The mRNA differential display technique (22) was used to compare gene expression in cells cultured for 24 h in 1% or 20% oxygen. Each experiment was performed in triplicate to assure reproducibility. cDNA bands exhibiting differential intensities were excised from the gels, cloned, and used as probes for Northern blotting.

One particular clone appeared to be markedly up-regulated in all differential display gels examined. Upon sequence analysis, the isolated 312-bp cDNA fragment displayed 99–100% similarity to a human gene previously identified by three separate groups, termed RTP (reducing agent and tunicamycin-responsive protein) (26), Drg1 (differentiation-related gene 1) (27), and Cap43 (28). Furthermore, the full cDNA sequence of the gene has been resolved and found to consist of 3056 base pairs that encode a 43-kDa protein (26).

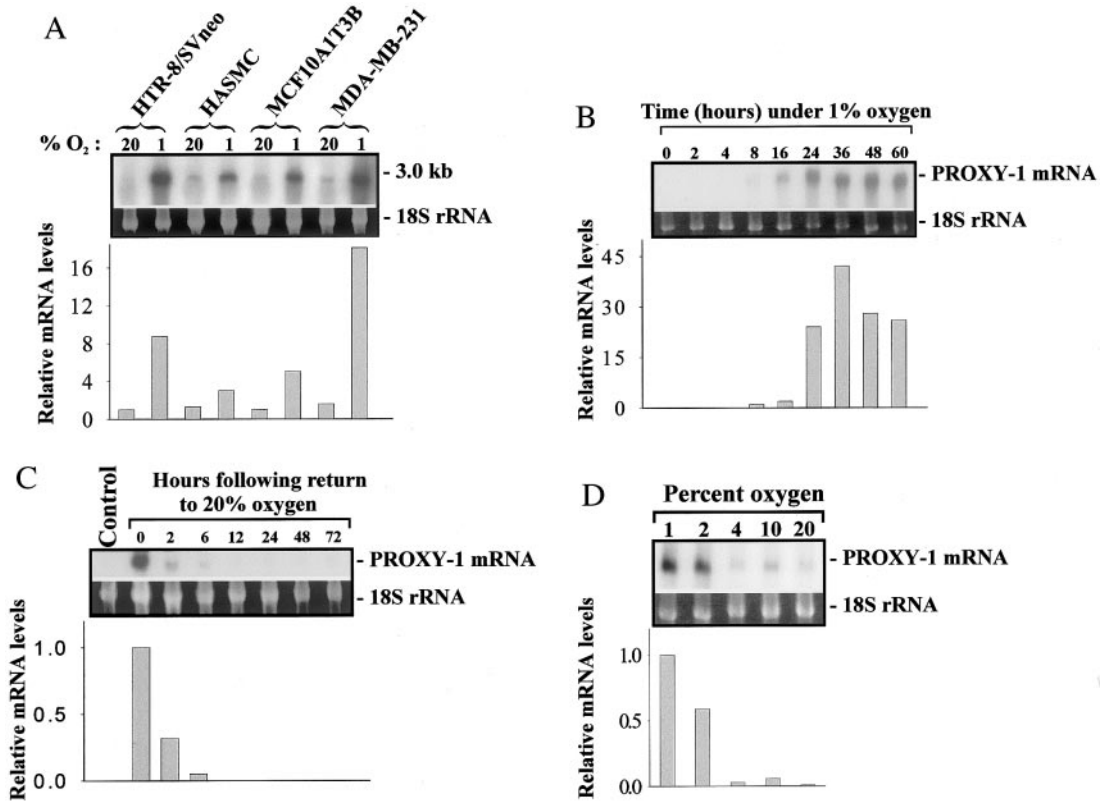

FIG. 1. Effect of hypoxia on the levels of PROXY-1 mRNA as determined by Northern blot analysis. Human first trimester trophoblasts (HTR-8/SVneo), aortic smooth muscle cells (HASMC), as well as MCF10A1T3B and MDA-MB-231 breast carcinoma cells were cultured under either standard (20% oxygen) or hypoxic (1% oxygen) for 24 h prior to analysis of PROXY-1 mRNA expression (A). Time-course assessment of PROXY-1 transcript expression in MDA-MB-231 cells (B). To determine the effect of reoxygenation on PROXY-1 mRNA levels, cultures of MDA-MB-231 cells were placed in a standard incubator at 20% oxygen following a 24-h incubation in 1% oxygen (C). To determine the amount of oxygen required to upregulate PROXY-1 mRNA expression, MDA-MB-231 cells were placed in 1%, 2%, 4%, 10%, and 20% oxygen for 24 h in a multi-gas incubator (D). Ethidium bromide staining of 18S rRNA was used to indicate the relative amount of RNA loaded in each lane. The experiments shown in this figure were repeated at least twice and similar results were obtained each time.

Effect of exposure to hypoxia on PROXY-1 mRNA levels. Northern blot analysis revealed that, following hypoxic culture, the levels of PROXY-1 mRNA were substantially increased (over 16-fold) in the HTR-8/SVneo trophoblast cells, the MDA-MB-231 and MCF10A1T3B breast carcinoma cells, and in the human aortic smooth muscle cells (Fig. 1A). The induction of PROXY-1 mRNA in MDA-MB-231 cells was first apparent after 8 h of exposure to hypoxia, reached a maximum at 36 h, and remained high for at least 60 h (Fig. 1B). Viability of the MDA-MB-231 cells incubated in 1% oxygen, as assessed by trypan blue exclusion and LDH accumulation, also remained high (>90%) and similar to that of control (20% oxygen) cultures for at least 48 h (data not shown). Following incubation of MDA-MB-231 cells for 24 h in 1% oxygen, a 2-h re-exposure to standard (20% oxygen) conditions was sufficient to substantially reduce PROXY-1 mRNA levels (Fig. 1C). Oxygen dose–response analysis revealed that, compared with culture in 20% oxygen, PROXY-1 mRNA levels were markedly increased in cells cultured in 1% or 2% oxygen for 24 h (16- and 10-fold, respectively; Fig. 1D). In contrast, no significant increases in PROXY-1 transcript levels were observed in cultures incubated in 4% oxygen or higher (Fig. 1D).

Expression of PROXY-1 protein. The PROXY-1 antiserum recognized a protein of the expected 43-kDa mass in Western blots. An increase in PROXY-1 protein was first apparent in MDA-MB-231 cells following 8-h culture in 1% oxygen (Fig. 2A). However, the highest levels of the 43-kDa protein were observed in cells cultured in 1% oxygen for at least 24 h (Fig. 2A). Although incubation for 24 h in 4% oxygen resulted in a small increase in protein levels, highest levels of expression were observed in cells cultured in 1% or 2% oxygen (Fig. 2B). Interestingly, PROXY-1 mRNA levels in cells incubated in 4% oxygen were similar to those in cells incubated in 20% oxygen (Fig. 1D). Furthermore, while PROXY-1 mRNA levels returned to control values soon after re-exposure to higher oxygen levels, reoxygenation did not result in any substantial drop in the levels of the 43-kDa protein for at least 12 h (Fig. 2C). Together, these observations suggest that the

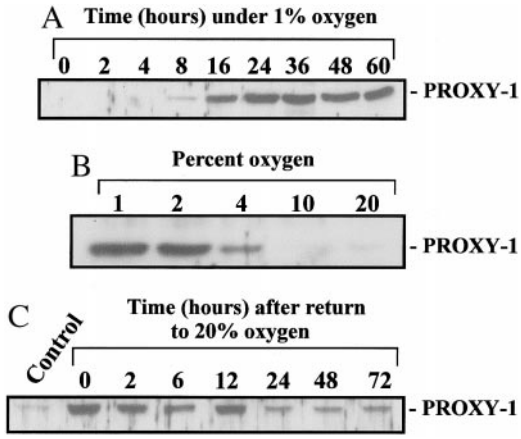

FIG. 2. Western blot analysis of PROXY-1 protein levels in MDA-MB-231 cells cultured in 1% oxygen for up to 60 h (A); cultured for 24 h under various levels of oxygen (B); and cultured for 24 h in 1% oxygen and then returned to 20% oxygen for up to 72 h prior to protein extraction (C). The data in this figure are representative of two or more independent experiments showing similar results.

half-life of PROXY-1 protein is longer than the half-life of its transcript. They may also explain the fact that PROXY-1 mRNA and protein levels do not always correlate.

Assessment of the role of a heme protein in the hypoxic regulation of PROXY-1 expression. To determine whether hypoxic responses are mediated through iron-containing heme proteins, the "deoxy" (hypoxic) conformation of such proteins can be maintained, even in the presence of oxygen, by replacing the ferrous iron atom within the porphyrin ring of the heme moiety with metals that do not bind oxygen or do so with very low affinity, such as cobalt. Alternatively, the function of heme proteins may be blocked through the use of iron chelators such as Tiron or desferrioxamine (DFO). Finally, carbon monoxide, which like oxygen binds to heme proteins and maintains them in the "oxy" conformation, can be used to interfere with heme protein-mediated hypoxic responses. Culture of MDA-MB-231 cells for up to 72 h in 20% oxygen with either 100 μM cobalt chloride, 30 mM Tiron, or 100 μM DFO resulted in time-dependent increases in PROXY-1 protein (Fig. 3A) and mRNA (Figs. 3B, 3C, and 3D). Furthermore, presence of 30% carbon monoxide in the hypoxic atmosphere reduced the hypoxic upregulation of PROXY-1 mRNA by 88% (Fig. 4A) and protein by 57% (Fig. 4B). To rule out the possibility that the inhibition of the hypoxic effect by carbon monoxide was not due to nonspecific toxicity, we cultured MDA-MB-231 cells with cobalt chloride, Tiron and DFO under hypoxic conditions in the presence of carbon monoxide. Western and Northern blot analysis revealed that the presence of carbon monoxide in the atmosphere did not inhibit the upregulation of PROXY-1 expression induced by cobalt chloride, Tiron and DFO (Figs. 4A and 4B).

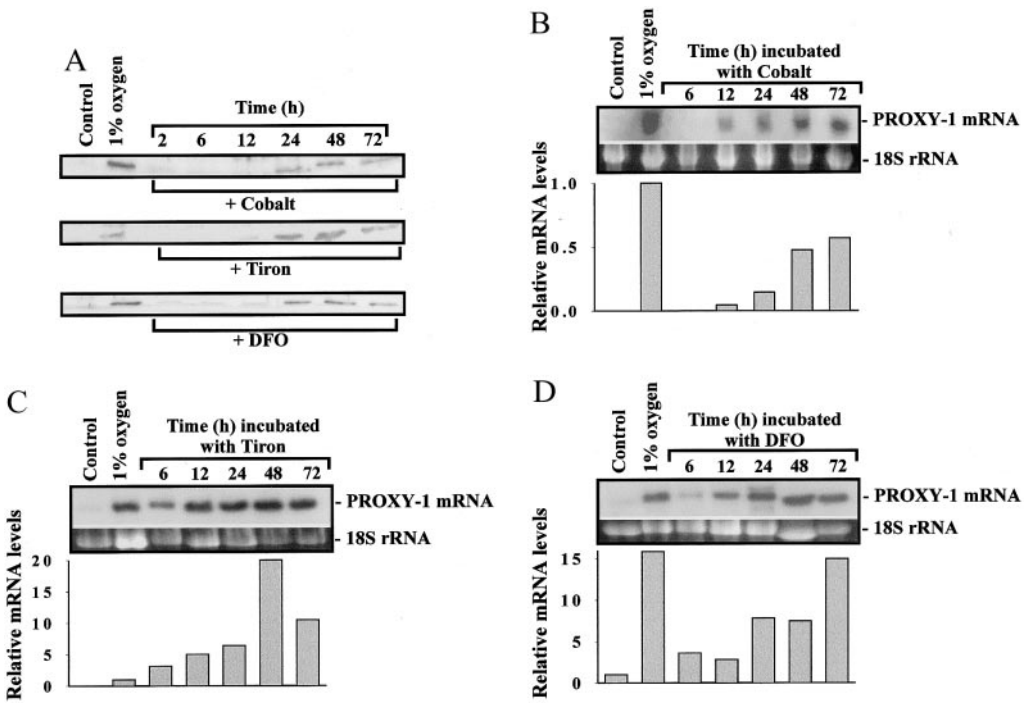

FIG. 3. Effect of cobalt chloride, Tiron, and desferrioxamine mesylate (DFO) on the levels of PROXY-1 protein (A) and mRNA (B, C, and D) in MDA-MB-231 cells. Cells were cultured for up to 72 h in the presence of the chemicals. The data in this figure are representative of three independent experiments exhibiting similar results.

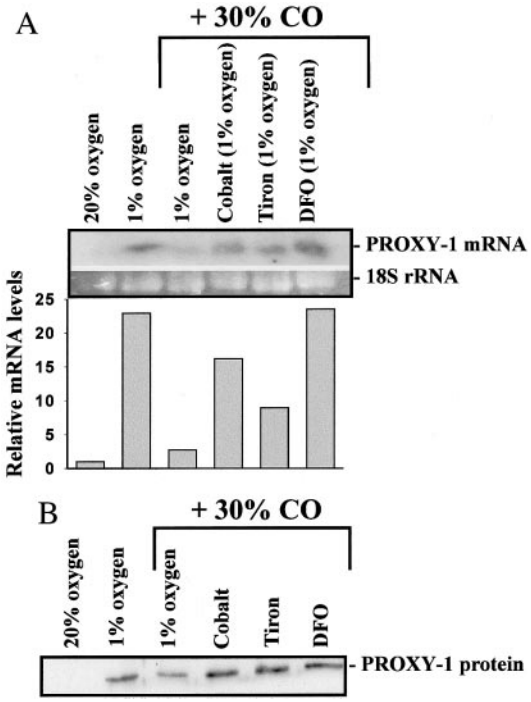

FIG. 4. Northern and Western blot analysis of PROXY-1 mRNA (A) and protein (B) expression by MDA-MB-231 cells cultured either alone in 20 or 1% oxygen or in 1% oxygen in the presence of carbon monoxide, with or without cobalt, Tiron and DFO. While the hypoxic upregulation of PROXY-1 mRNA and protein expression was reduced by inclusion of 30% CO in the hypoxic atmosphere, CO was unable to inhibit the upregulation of PROXY-1 expression induced by cobalt chloride, Tiron, and DFO, even under conditions of low oxygen.

DISCUSSION

The major findings of the present study demonstrate the upregulated expression of a gene (*PROXY-1*) in various cell types cultured under low levels of oxygen. They also reveal that the hypoxic increases in PROXY-1 mRNA and protein levels are time-dependent, showing detectable elevations at approximately 8 h of hypoxic culture, and that protein levels remain high long after cessation of the hypoxic stimulus. Furthermore, a direct correlation between the extent of hypoxia, within physiologically relevant values, and PROXY-1 protein and mRNA levels was observed. The expression of PROXY-1 mRNA (as detected from total cellular RNA extracted) and protein is almost completely undetectable at 10% oxygen or higher levels but displays marked upregulation at 1 or 2% oxygen.

Other studies have shown increased expression of PROXY-1 (or identical genes) in homocysteine-treated endothelial cells (26), colon epithelial cells (27), and in human lung A549 cells exposed to nickel (28). In a recent study, PROXY-1 expression was found to be diminished in a variety of cultured tumor cells when compared with their normal counterparts (29). Moreover, PROXY-1 (named RTP/rit42 in that study) expression was shown to be upregulated by DNA-damaging agents in a p53-dependent manner, and its overexpression in human tumor cells resulted in reduced cell growth both *in vitro* and in nude mice (29). In addition to hypoxia, the expression of PROXY-1 is increased in response to other potentially harmful stimuli, such as exposure to cytotoxic agents (28) and glucose deprivation (H. Park and C. H. Graham, unpublished observations). Thus, it is possible that the 43-kDa PROXY-1 protein plays a role in protecting cells from such insults. Although other potential functions of the 43-kDa protein encoded by this gene have not been determined, examination of the deduced amino acid sequence reveals the absence of a signal sequence, transmembrane domains, or metal-binding domains (28). The presence of a putative phosphopentetheine attachment site and a novel motif of three-tandem repeats (GTRSRSHTSE) at the C-terminal domain suggests that it may belong to a novel family of polypeptides. The fact that the 43-kDa protein exhibits 93% homology to a murine protein encoded by the *Ndr1* gene (30) and 28% homology to a *C. elegans* protein, ZK1073.1 (31) indicates a high degree of conservation and a likely vital role. Furthermore, alignment of the amino acid sequences of the human and mouse proteins reveals conservation in all eight cysteine residues, suggesting a similar three-dimensional conformation. Table 1 summarizes the relationship of PROXY-1 to other proteins.

Our results suggest that the hypoxic upregulation of PROXY-1 is mediated through a putative heme protein. This conclusion is derived from experiments in which the ability of such a protein to regulate PROXY-1 expression was assessed through the use of cobalt chloride, Tiron and DFO, which have traditionally been shown to mimic hypoxia by preventing the binding of oxygen (and/or other ligands) to heme groups, or by blocking heme biosynthesis. The involvement of an iron-containing heme protein in the hypoxic upregulation of several genes, including erythropoietin (7), vascular endothelial growth factor (32, 33), and others (34), has been determined using similar approaches. In our study, incubation with these three compounds led to similar observations, suggesting that the findings were due to inhibition of a heme-containing protein and not a result of non-specific effects. Furthermore, we showed that the upregulation of PROXY-1 expression in response to hypoxia was inhibited by carbon monoxide. The fact that carbon monoxide was unable to block the upregulation of PROXY-1 expression induced by cobalt chloride, Tiron and DFO, also demonstrates that its effect was not due to non-specific toxicity. Interestingly, when the PROXY-1 gene was cloned independently by Zhou *et al.* (28) (named Cap43 by these investigators), it was found to be induced by nickel, a molecule that also replaces

TABLE 1

Similarities between PROXY-1 and Related Proteins

Name	Reference	Species	% Protein identity to PROXY-1[a]	Description
RTP[b]	(26)	Human	99	Homocysteine-responsive gene in vascular endothelial cells
Drg1[c]	(27)	Human	100	Differentiation-regulated gene in colon epithelial cells
Cap43	(28)	Human	99	Induced by Ni^{2+} compounds in lung epithelial cells
rit42	(29)	Human	100	Induced in tumor cells by DNA-damaging agents
Ndr1	(30)	Mouse	93	N-myc-downstream gene
TDD5	(38)	Mouse	75	Androgen target gene repressed by testosterone and dihydrotestosterone
ZK1073.1	(31)	*C. elegans*	28	Nucleotide sequence from chromosome III of *C. elegans*
SF21	(39)	Sunflower	29	Transmitting tissue and pollen-expressed protein

[a] BLAST multiple sequence alignment.
[b] GenBank Accession No. NM006096.
[c] GenBank Accession No. X9284.

ferrous iron from heme proteins. It is possible that, in their study, the upregulation of PROXY-1 in lung epithelial cells in response to nickel exposure was in part due to the heme protein-blocking properties of nickel. There is also evidence that, in addition to acting at the level of iron-containing heme proteins, compounds like cobalt and DFO mimic hypoxia by preventing proteasome-mediated degradation of HIF-1 and HIF-2 through a process that involves dissociation of HIF-1 and HIF-2 from the von Hippel-Lindau tumor suppressor gene product (16).

The present findings demonstrated substantially increased PROXY-1 expression in cells incubated in 1% and 2% oxygen compared to cells cultured at or above 4% oxygen. These results may be of physiologic and clinical relevance, as most tissues in the body are exposed to oxygen concentrations equivalent to 5% or lower. Our study also showed that the high levels of expression of PROXY-1 protein persisted for at least 72 h following a hypoxic stimulus, indicating a relatively slow turnover of this protein. In contrast to this, we have found that the increased expression of cell surface urokinase receptors in response to hypoxia returns to background levels following reoxygenation for as little as 45 min (T. E. Fitzpatrick and C. H. Graham, unpublished observations). The fact that cellular PROXY-1 protein levels remain high for an extended period following transient exposure to low levels of oxygen may also be relevant to conditions in which relatively short periods of exposure to hypoxia occur, such as during temporary vasoconstriction, sleep apnea, or fetal hypoxia. Our findings suggest that such episodes of hypoxia may lead to long-lasting phenotypic changes that could persist well beyond the hypoxic insult and into the period of normoxia. The clinical consequences of this persistent phenotype need to be further elucidated. Since our results show a direct correlation between the extent of hypoxia and PROXY-1 expression, assessment of the levels of this protein may be useful in determining the extent of oxygen deprivation in the above settings. In addition, studies have revealed an association between low intratumoral oxygen levels and poor clinical outcome for patients with various types of cancers (35–37). The 43-kDa protein may therefore be of prognostic value as a marker of hypoxic regions within the tumor mass. We have preliminary data that show increased PROXY-1 immunostaining in the hypoxic, deeper layers of three-dimensional aggregates of HTR-8/SVneo cells and MDA-MB-231 cells compared with oxygenated superficial layers (H. Park, M. A. Adams, and C. H. Graham, unpublished observations). Furthermore, studies in our laboratory have revealed increased mRNA levels in peri-infarct regions of placentae of preeclamptic women compared to non-infarct areas in similar placentae or normal placentae (H. Park, V. K. Han, R. Gratton, and C. H. Graham, unpublished results).

In summary, our new findings demonstrate the hypoxic induction of PROXY-1 expression over a physiologically relevant range of oxygen levels. Using the methods described, PROXY-1 expression was undetectable in cells cultured under standard conditions but its levels increased substantially in cells exposed to 1% or 2% oxygen. Together with the fact that this gene is expressed by a variety of unrelated cell types, this type of gene expression suggests that it may be a universal 'switch' involved in cellular adaptation to hypoxia.

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