Hypoxia-enhanced Expression of the Proprotein Convertase Furin Is Mediated by Hypoxia-inducible Factor-1

IMPACT ON THE BIOACTIVATION OF PROPROTEINS*

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Hypoxia is a common tumorigenesis enhancer, mostly owing to its impact on gene expression of many angiogenic and invasion-related mediators, some of which are natural substrates for the proprotein convertase furin. Analysis of furin promoters revealed the presence of putative binding sites for hypoxia-inducible factor-1 (HIF-1), a transcription complex that plays a pivotal role in cellular adaptation to hypoxia. In fact, we demonstrate herein that the levels of fur mRNA, encoding furin, are remarkably increased upon hypoxic challenge. Cotransfection of a HIF-1α dominant negative form in wild-type (WT) cells or transfection of a furin promoter-reporter gene in HIF-1-deficient cells indicated the requirement of HIF-1 for furin promoter activation by hypoxia. Direct HIF-1 action on the furin promoter was identified as a canonical hypoxia-responsive element site with enhancer capability. The hypoxic/HIF-1 regulation of furin correlated with an increased proteolytic activation of the substrates membrane-type 1 matrix metalloproteinase and transforming growth factor-β1. Our findings unveil a new facet of the physiological consequences of hypoxia/HIF-1, through enhanced furin-induced proteolytic processing/activation of proproteins known to be involved in tumorigenesis.

The induction of angiogenesis and cell invasion by hypoxia is a hallmark of pathological processes such as wound healing, arthritis, and solid tumor formation. Oxygen deprivation induces gene expression of many polypeptides that participate in the establishment of new blood vessels and in the invasion phenotype (1–4). Several of these proteins are first synthesized as inactive propeptides that require maturation through limited endopeptidolytic cleavage after a sequence of two or more basic residues (Lys or Arg) to acquire activity. Among them are growth factors such as transforming growth factor-β1 (TGFβ1), platelet-derived growth factor, insulin-like growth factor, and one of its receptor (insulin-like growth factor and insulin-like growth factor receptor-1), hepatocyte growth factor, metalloproteinases, including membrane-type 1 matrix metalloproteinase (MT1-MMP), and the homotypic cell-cell interaction molecules E-cadherins and the integrin αvβ3 (3, 5–14). In the past decade, a novel family of seven closely related mammalian subtilisin/kexin-like serine proteases with this cleavage specificity was discovered. They are grouped under the generic name of proprotein convertases (PCs) and include PC1/PC3, PC2, PC4, PC5/PC6, PC7, and PACE4. Within this family, selected members exhibit a tissue-specific distribution, such as PC1, PC2, and PC4, whereas furin, PACE4, PC5/PC6, and PC7 are expressed in a broad range of tissues and cell lines (for review see Refs. 15 and 16). Among these convertases, furin is the first and so far the best-characterized enzyme. The biological importance of this PC arises from the large number and variety of bioactive proteins and peptides that can be generated through its activity, including key elements involved in normal and pathophysiological conditions such as cancer.

Recent studies indicate that tumor growth and malignant tumor phenotypes are regulated by the action of PCs, especially furin. In fact, inoculation of immunodeficient mice with furin-inhibited cells resulted in delayed and lower incidence of tumor development as well as reduced tumor size compared with wild type (WT) cells. Interestingly, these observations were accompanied by a significant decrease in tumor vascularity (14). In normal tissues, furin is detectable at very low levels, however, elevated expression of this convertase has been reported in a variety of human cancers, including breast tumors, head and neck tumors, glyoblastomas, and lung cancer (17–20). Furin expression has also been correlated with cancer aggressivity and was therefore proposed to have significant prognostic value (21). This suggests that the potentially deleterious effects of furin on the maintenance of cellular homeostasis under physiological conditions are avoided by very low cellular levels of expression.

The mechanisms by which the fur gene, encoding furin, is differentially expressed and regulated in tumor conditions are still poorly understood. It is known that at least three distinct promoters, namely P1, P1A, and P1B, direct its transcription (22). The fur transcripts generated differ in their 5′-end but are all translated from the same AUG, giving rise to identical furin proteins. The P1A and P1B promoters resemble convertase; WT, wild-type; HRE, hypoxia responsive element; HIF-1, hypoxia-inducible factor-1; HBS, HIF-1 binding sequence; HAS, HIF-1 ancillary sequence; MEM, minimal essential medium; v1-PDX, v1 anti-trypsin Portland; EMSA, electrophoretic mobility shift assay; TK, thymidine kinase; DN, dominant negative; PACE, paired basic amino acid-converting enzyme.
HIF-1-dependent Hypoxic Regulation of Furin

Background

Furin is a proprotein convertase that is predominantly expressed in the pancreas and testis, and its expression is regulated by hypoxia. The enzyme plays a crucial role in the processing of numerous prohormones and proenzymes. In this study, we aimed to understand the mechanism by which hypoxia regulates furin expression.

Materials and Methods

Cell Culture

The hepatoma cell lines HepG2 (human) and Hepa-1 c1c7 (mouse) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The Hepa-1 c4 cells, derived from Hepa-1 c1c7 (mouse) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The Hepa-1 c4 cells were plated at a density of 125,000 cells/well in 6-well plates. Cell lines were transfected with CaPO4 precipitation technique using a Mammalian Cell Transfection Kit (Speciality Media, Lavallette, NJ) as described previously (46). Briefly, twenty-four hours prior to transfection, cells were plated at a density of 125,000 cells/well in 6-well plates. Cells were transfected with 2–4 μg of plasmids/well, as indicated in the figure legends. Twenty hours following transfection, cells were serum-starved 3–4 h prior to overnight exposure to the hypoxic or normoxic environment. Cell lysates were assayed for luciferase activity as described previously (47). Control pGL2-Basic vector was routinely used as an internal control of transfection. Values were normalized for transfection efficiency as described previously (23, 47).

Site-directed Mutagenesis

The HIF-1- and H5-HRE sequences located at position −1011 and −885, respectively, within the P1 promoter were mutated by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Distinct mutations were generated by replacing the H4 motif 5′-TAGTGAC-3′ by 5′-TACGACC-3′ and/or the H5 motif 5′-GAGGTGTTGGTGCACCC-3′ by 5′-GAGCAACGTTGGCTGCTTC-3′. Each mutation was verified by direct sequencing.

Plasmid Construction

The oligonucleotides encoding four tandem repeats containing the wt H5-HRE sequence 5′-GACCGGGCGTGGTG-3′ and the wt H5-HRE sequence 5′-TATGTGC-3′ were subcloned into the plasmid pGL3-TK-basic vector (generously provided by Dr. Negishi, National Institutes of Health, Research Triangle Park, NC).

Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared as described previously with few modifications (48, 49). Briefly, 5 μg of nuclear extracts was incubated with 4 × 10^6 cpm of [32P]-labeled double-stranded oligonucleotides in modified Z-100 buffer (50 mM HEPES, 15 mM dithiothreitol, and 0.1 mM of calf thymus DNA/20 μl). The sequences of the sense strands of the oligonucleotides used for EMSA were as follow: 5′-CGGCGACCGGGCGGTGGGTGGCCACGTGAAAGGCTCGTGTGGGGTCCGG-3′ (H1), 5′-GAGGTGTTGGTGCACCC-3′ (H5-HBS). Mouse anti-HIF-1α (Novus Biologicals, Littleton, CO) and isotype-matched (Sigma) antibodies were used in supershift assays.

Northern Blot Analysis

RNA was isolated from control and hypoxic cells as previously described (30–35). The 3′-UTR of the P1 promoter was subcloned into the plasmid pCDNA3-HIF-1α. Distinct mutations were generated by replacing the H4 motif 5′-TAGTGAC-3′ by 5′-TACGACC-3′ and/or the H5 motif 5′-GAGGTGTTGGTGCACCC-3′ by 5′-GAGCAACGTTGGCTGCTTC-3′. Each mutation was verified by direct sequencing.

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Results

Kineti cs of Furin, PACE-4, and PC7 Expression following Hypoxia Exposure—Hypoxia is a common tumorigenesis enhancer, mostly owing to its impact on gene expression of many angiogenic and invasion-related mediators, some of which are natural substrates for the convertase furin. Because of the emerging role of protoprotein convertases (furin) in tumorigenesis, we postulated that furin could be regulated by hypoxia to achieve adequate proteolytic maturation of angiogenic and/or tumorigenic substrates. To test this hypothesis, HepG2 cells

Conclusion

The results of this study indicate that hypoxia regulates furin expression through a mechanism involving the transcriptional activation of the P1 promoter. This finding is significant as it sheds light on the role of hypoxia in the regulation of furin expression and its potential implications in tumorigenesis. Further studies are needed to elucidate the precise mechanisms by which hypoxia modulates furin expression and to explore therapeutic strategies targeting furin expression in hypoxic microenvironments.
were cultured in normoxic (21% O₂) or hypoxic (1% O₂) conditions for time periods ranging from 4 to 24 h. Results expressed in Fig. 1 indicate that hypoxia is a strong inducer of furin mRNA accumulation in HepG2 cells with a maximum increase of 18-fold observed at 24 h, whereas PACE-4 and PC7 mRNA levels were mostly unaffected. Extended cell culture in hypoxic conditions (48 h) resulted in no additional increase in furin mRNA levels (data not shown). These results indicated that furin, but not the other convertases PACE-4 or PC7, is a hypoxia-inducible gene.

A similar increase in furin mRNA by hypoxia was also observed in other cell lines, including RAW 264.7 mouse macrophages, primary rat synoviocytes, as well as mouse Hepa-1 c1c7 hepatoma cell line (data not shown). These results indicated that furin, but not the other convertases PACE-4 or PC7, is a hypoxia-inducible gene.

A different increase in furin mRNA by hypoxia was also observed in other cell lines, including RAW 264.7 mouse macrophages, primary rat synoviocytes, as well as mouse Hepa-1 c1c7 hepatoma cell line (data not shown). This indicates that the regulation of the furin gene by hypoxia is extended to various cell types and species.

**HIF-1 Is Required For the Hypoxic Response of the fur P1 Promoter**—Computer-assisted analysis of fur promoter sequences revealed the presence, within all three fur promoters, of putative HIF-1 binding sites (HBS), including several closely spaced HIF-1 ancillary sequences (HAS) characteristic of HIF-1-regulated genes (Fig. 2A) (37). This suggested that HIF-1 may regulate fur promoter activity under hypoxic conditions.

To address this possibility, HepG2 cells were transiently transfected with either P1, P1A, or P1B promoter-Luc constructs, or with the promoter-less vector pGL2-Luc. Cells were incubated overnight under normoxic or hypoxic conditions before luciferase activity measurement. The induction ratio of the luciferase activity by hypoxia is indicated for each luciferase promoter construct. Data are expressed as the mean ± S.E., n = 3. *, p < 0.05, compared with normoxic control.

**FIG. 1.** Expression of furin in hypoxic conditions. Kinetics of fur, PACE-4, and PC7 mRNA accumulation. HepG2 cells were cultured in normoxia (21% O₂) or hypoxia (1% O₂) for various time periods as indicated. Total mRNA (5 μg/lane) was probed with specific furin, PACE-4, and PC7 rat riboprobes. A 18 S probe was used as an internal control. The autoradiogram and the densitometry ratio of each convertase/18 S (controls set to 1) are represented.

**FIG. 2.** Effect of hypoxia on fur P1, P1A and P1B promoter activity. A, schematic representation of localization and sequence of the HRE motifs present in the 5′ noncoding exons 1, 1A, and 1B of the human fur gene. B, transient transfection of HepG2 cells with 2 μg/well pGL2-Basic (promoterless vector), pGL2-P1, pGL2-P1A, or pGL2-P1B promoter constructs. Cells were incubated overnight in normoxic or hypoxic conditions before luciferase activity measurement. The induction ratio of the luciferase activity by hypoxia is indicated for each luciferase promoter construct. Data are expressed as the mean ± S.E., n = 3. *, p < 0.05, compared with normoxic control.
Thus, the response of P1 promoter fragments to HIF-1 overexpression correlates with their response to hypoxia, suggesting that one or more hypoxia response elements within P1 promoter are HIF-1 binding elements.

To determine whether HIF-1 is indeed required for transcriptional activation of the furin P1 promoter fragment by hypoxia, we either cotransfected a dominant negative isoform of HIF-1α/H9251 in HepG2 cells or the P1-promoter-luc construct was transfected into the Hepa-1 c4 hepatoma cell line, which lacks HIF-1α/ARNT protein and thereby cannot form the HIF-1 transcription complex (51). Results in Fig. 4 (A and B) indicated that forced expression of the dominant negative HIF-1α isoform in HepG2 cells greatly reduced both P1 and P1-NheI transactivation in response to hypoxic challenge. Similarly, the P1 luciferase reporter gene was activated in the parental Hepa-1 c1c7 cell line but not in the HIF1α/ARNT-deficient Hepa-1 c4 cells (Fig. 4C). Together, these results clearly demonstrate the requirement of HIF-1 for the hypoxic activation of the furin P1 promoter. Results from the dominant negative experiment also suggest that the H4- and/or H5-HRE sites within the P1-NheI promoter fragment are functional.

**Functional Analysis of HIF-1 Binding Sites within the P1-NheI Promoter Fragment**—To define the functionality of the HRE sites in the P1-NheI promoter fragment, site-directed mutagenesis was performed on the two putative H4- and H5-HRE sites. This resulted in three distinct mutants, P1-Nhe1H4 MUT, P1-Nhe1H5 MUT, and P1-Nhe1H4/H5 MUT, that correspond to elimination of the H4-HRE, H5-HRE, or both sites, respectively. Expression of the plasmid encoding the P1-Nhe1H4 MUT (Fig. 5A) resulted in a similar luciferase activity in response to HIF-1 as the WT P1-NheI, whereas mutation of the H5 site reduced HIF-1 stimulation to 10% of the WT fragment, indicating that this site is critical for the HIF-1 response. Mutation of both H4 and H5 sequences did not result in additional inhibition, indicating that these sites do not act in a cooperative manner.

To investigate whether the H5-HRE site within the furin P1 promoter has enhancer activity in response to HIF-1, four tandem repeats of a 28-nucleotide sequence that encompasses both the HBS and the HAS of the H5-HRE binding site (4XH5 fur-P1 WT), or a mutant sequence (4XH5 fur-P1 MUT) with a 6-nucleotide substitution that eliminates both the HBS and the HAS sites, were cloned upstream of the minimal thymidine kinase (TK) promoter and the luciferase reporter gene. Luciferase activity assays of the resulting WT construct indicated an 8.9-fold increase in the presence of coexpressed HIF-1 (Fig. 5B), whereas the activity of the mutant construct remained close to basal value. Taken together, these results demonstrate that the H5-HRE site present in the furin P1 promoter is critical for HIF-1-induced enhancer activity.
To ensure that the identified H5-HRE site could physically bind HIF-1, we performed electrophoretic mobility shift assays using a 28-bp probe containing the WT H5-HBS and HAS sites. Oligonucleotides encoding the H4 site were also used for comparison. Three retarded bands (R1, R2, and R3) were obtained with nuclear extracts from hypoxic cells (Fig. 6, lane 2), whereas nuclear proteins from normoxic cells showed only one major band of constitutive binding activity (lane 1). In contrast, no hypoxia-inducible complexes were observed when using the H4 oligonucleotide (lanes 9–11). All retarded complexes demonstrated specificity in their binding to H5 WT sequence, because their efficiency was competed using a 100-fold molar excess of unlabeled oligonucleotides (wt; lane 5). In contrast, no competition was observed with oligonucleotides containing mutations to eliminate the H5-HBS and HAS sites (mut, lane 6). Also, no hypoxia-driven complexes were detected when the H5 mut oligonucleotide was used as a radiolabeled probe (lanes 7 and 8). An anti-HIF-1α antibody supershifted the R1, R2, and R3 complexes into two slower migrating bands (lane 3), whereas an isotype-matched control antibody was inefficient (lane 4). This indicates that HIF-1 is a component of the three retarded complexes.

Results shown in Fig. 3B suggested that the most distal promoter fragment encompassing the two putative H1- and H2-HRE sites may participate in P1 induction by hypoxia, because its deletion slightly reduces the overall response. To test this possibility, the H1 and H2 oligonucleotides were included in a parallel EMSA assay (lanes 12–17). Results indicate the absence of hypoxia-induced complexes for both probes, when the autoradiograms were revealed within standard (16 h) exposure time. However, longer exposures resulted, for the H1 probe, in a similar pattern of hypoxia-induced and antibody supershifted complexes, as observed with the H5 probe (data not shown). This suggested that weak interactions between HIF-1 and the H1 site can occur, in addition to the stronger interactions detected in the case of the H5 site.

Recent data demonstrate in several HIF-1-inducible genes a common HRE structure that consists of a HAS and a HBS site (37, 52–54). Because H5 contains both sites, we investigated whether the HBS site is sufficient for HIF-1 interaction. To this end, a probe containing only the H5-HBS site was radiolabeled and tested in EMSA. Results in Fig. 6 (lanes 18–20) indicate that removal of the HAS site impairs HIF-1 binding to the H5-HRE sequence, indicating that both the HAS and HBS are indeed required.

**Impact of Increased Furin Expression on Substrate Maturation**—We investigated whether furin mRNA levels correlate with the extent of proteolytic conversion of the furin substrates, MT1-MMP and TGFβ1. These substrates contain a proven furin activation site, and both have been shown to profoundly affect many aspects of tumor progression (7, 11, 12, 24, 55). We therefore analyzed by immunoblotting MT1-MMP-related digestion products obtained from HepG2 hepatoma cells, which express high levels of endogenous MT1-MMP. As shown in Fig. 7A, a high molecular mass band (66 kDa) corresponding to the unprocessed MT1-MMP precursor is observed in both culture conditions. Hypoxia resulted in an augmented MT1-MMP production, as well as in the conversion of the MT1-MMP precursor into the 60-kDa mature form. For TGFβ1 maturation, we analyzed by Western blotting lysates from the Hepa-1 c1c7 hepatoma cell line overexpressing this growth factor (to permit immunodetection). As demonstrated in Fig. 7B, hypoxia-treated cells exhibited, in addition to the 55-kDa precursor band, a 44-kDa product corresponding to the proteolytically processed prodomain product (7).

In the same experiment, we studied whether hypoxia-induced increase in TGFβ1 maturation by furin is mediated by HIF-1. For this, TGFβ1 digestion products were analyzed in the derived HIF-1-deficient Hepa-1 c4 clone and compared with those obtained with the control Hepa-1 c1c7 cells. Results indicated that only the HIF-1-proficient Hepa-1 c1c7 cells produced a mature TGFβ1 species under hypoxic condition, whereas such a maturation product was absent in the c4 clone. In parallel, the increased production of active TGFβ1 under hypoxia was restricted to the Hepa-1 c1c7 cells expressing the HIF-1 complex (Table I). These results indicate that hypoxia-induced TGFβ1 endoproteolysis by furin is indeed regulated by endogenous HIF-1. Thus, hypoxia/HIF-1-induced endogenous fur gene expression correlates with an increase in MT1-MMP and TGFβ1 endoproteolysis.

Previous data obtained in our laboratory clearly defined furin as an authentic transforming growth factor-β1-converting enzyme (24). To ensure that the proteolytic maturation of TGFβ1 in hypoxic conditions is achieved by furin, we overexpressed TGFβ1 and the described previously furin inhibitor α1-PDX in Hepa-1 c1c7 and c4 cell lines (56). As demonstrated in Fig. 7C, the production of the mature form of TGFβ1 observed in hypoxia-treated WT c1c7 clone was inhibited upon α1-PDX expression. In parallel, hypoxia-induced release of bio-
active TGFβ1 in Hepa-1 c1c7 cell-conditioned media was blunted to control normoxic cells level following the expression of P1-NheI with measured amounts of 4.44 ng/ml versus 0.5 ng/ml, respectively. Thus, furin is the enzyme responsible for the hypoxia-induced conversion of pro-TGFβ1 into its bioactive form.

**DISCUSSION**

In this report, we provide evidence that the fur gene is induced upon hypoxia exposure, and we identified a region within its promoters that regulates furin transcription under hypoxia, through HIF-1 activity. More significantly, we demonstrate that such hypoxic/HIF-1 regulation of furin correlated with an increased proteolytic activation of the substrates MT1-MMP and TGFβ1. Our study revealed a new facet for the role of HIF-1 in tumor progression, through enhanced proteolytic processing/activation of proproteins.

Three alternative promoters can drive the transcription of the fur gene (22). Promoters P1A and P1B resemble housekeeping genes with multiple Sp1 binding sites. However, the P1 promoter has features of the inducible class (22, 23, 47). Herein, we demonstrate that even though each of the three fur promoters can respond to hypoxia, the P1 promoter is the most sensitive to oxygen deprivation, with an average 3-fold increase (compared with 1.8- and 1.4-fold increases for P1A and P1B, respectively). Analysis of the strong hypoxia-inducible P1 promoter revealed that deletion of the H4- and H5-HRE sites located within the −1221 to −413 (P1-NheI) region strongly abolished the response to both hypoxia and HIF-1. The elimination of H4 did not affect P1-NheI activity toward HIF-1-forced expression, although the abolition of H5 site completely impaired the response to HIF-1, indicating that the integrity of H5 is crucial for P1 responsiveness to hypoxia. A four-tandem

![Diagram](image-url)
repeat of H5-HRE efficiently enhanced minimal thymidine kinase promoter activity toward HIF-1, indicating that this site also has enhancer activity. A common structure of the HRE consists of the core HIF-1 consensus binding sequence (HBS), 5’/H11032 (A/G)(C/T)GTG-3’/H11032, located upstream of an imperfect inverted repeat, 5’/H11032 -CAC(A/G)(T/C)-3’/H11032, called HIF-1 ancillary sequence (HAS), with a spacing of 8 or 9 nucleotides between the HBS and HAS, features crucial for its activity. This is the case for many HIF-1-regulated genes, including erythropoietin, vascular endothelial growth factor, and Glut-1 (37). Analysis of the H5-HRE site of the furP1 sequence indicated that it is constituted of both HBS and HAS sequences (5’/H11032 GCGTG GTGGCT-3’/H11032), identical to the Glut-1 HRE (5’/H11032 GCGTG CCGTCT-GA CACGC-3’), with the exception of a 6-nucleotide spacing instead of 8 nucleotides between the HAS and the HBS sites (37). In EMSA, a probe containing only the HBS site of H5-

TABLE I
Measure of bioactive TGFβ1 from hypoxia-stimulated Hepa-1 c1c7 and c4 cells

<table>
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<th>Experiments</th>
<th>Bioactive TGFβ1</th>
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<th>HIF-1-deficient C4</th>
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<td>N H Hypoxic increase</td>
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<td>2</td>
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FIG. 6. Analysis of HIF-1 binding to HREs within furP1 promoter. HepG2 cells were cultured 16 h in normoxia or hypoxia prior to the nuclear extracts preparation. The nuclear extracts were incubated with the wt H5 radiolabeled probe (wt H5, lanes 1–6). The specificity of complex formation was tested by the inclusion of 100-fold excess of unlabeled competitors in the binding buffer (cold probe (wt), lane 5; cold mutated H5 probe (mut), lane 6), by including a specific anti-HIF-1x antibody (a, lane 3) or an isotype-matched control antibody (c, lane 4), or by the use of radiolabeled mut H5 probe in EMSA (lanes 7 and 8). Radiolabeled probes containing only the HBS site of H5 (lanes 18–20) or including other HREs sequences within furP1 promoter were also tested for their binding to HIF-1 (H1, lanes 12–14; H2, lanes 15–17; and H4, lanes 9–11). S1 and S2 indicate supershifted complexes; C, constitutive band; R1, R2, and R3, HIF-1 binding complexes; and NS, nonspecific band.

FIG. 7. Biological impact of hypoxia/HIF-1-induced furin gene expression. Western blot analysis of furin processing of MT1-MMP and TGFβ1. A, HepG2 cells were cultured in normoxia (N) or hypoxia (H) for 16 h. B, Hepa-1 c1c7 and c4 cells were infected with adenovirus expressing green fluorescent protein only (CTL) and/or pro-TGFβ1 (TGFβ1) (C) in the presence or absence of the furin inhibitor expressing adenovirus (a1-PDX) at a final multiplicity of infection of 200 and cultured in normoxia or hypoxia for 16 h. Cell lysates were separated on 7.5% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Immunoblotting was performed using anti-MT1-MMP or anti-latency-associated peptide antibodies. Three immunoreactive MT1-MMP bands can be visualized corresponding to the pro form (66 kDa), the active form (60 kDa), and a degradation form (~45 kDa). Anti-LAP immunoblotting revealed two bands corresponding to the pro-TGFβ1 (55 kDa) and the pro-domain (44 kDa).
HIF-1-dependent Hypoxic Regulation of Furin

The proprotein convertase furin shares a similarity of cleavage site specificity with the six other members of the human proprotein convertases family, and consequently, redundancy in substrate processing often occurs (15). Most of the angiogenic/tumorigenic factors requiring bioactivation are processed within the constitutive secretory pathway, where the greatest part of the converting activity is achieved by furin, PACE-4, PC5/PC6, and PC-7 (24). Because an increased expression in PACE-4 and PC7 mRNAs was previously observed in different cancer types (18, 57, 58), and because both of these convertases are expressed along with furin in HepG2 cells (59), we analyzed the impact of hypoxia on their expression. Although furin expression was dramatically increased upon oxygen deprivation, PACE-4 and PC7 mRNA levels were only slightly modulated, indicating that hypoxia-induced expression is not extended to all human proprotein convertase family members. Nucleotide analysis of the 5′-flanking region of the human PACE-4 and PC7 genes demonstrated the absence of TATA and CAAT elements, promoter features characteristic of housekeeping genes (60, 61). However, the PACE-4 promoter contains putative binding sites for several transcription factors such as AP-1, growth hormone factor-1, and CREB (cAMP response element-binding protein) and was shown to be regulated by the growth factor, platelet-derived growth factor-BB (62). Further analysis of the cloned promoter portions for each of these convertases revealed the absence of a canonical HRE in PACE-4 and PC7 genes. Although several transcription factors have been reported to be activated in hypoxia, such as NK-X8 and AP-1 (63), HIF-1 was repetitively demonstrated to be the main regulator of the mammalian adaptive response to low oxygen tension. Therefore, the observed absence of HRE consensus HIF-1 binding sites may explain the lack of significant hypoxic response.

The hypoxia/HIF-1-dependent increase in furin gene expression was found to be involved in the increased bioavailability of angiogenic/tumorigenic mediators as exemplified by the metalloproteinase MT1-MMP and the growth factor TGFβ. Both mediators are well characterized furin substrates that have been shown to profoundly affect many aspects of tumor progression. MT1-MMP, through proteolytic events, regulates various cellular functions, including extracellular matrix turnover, promotion of cell migration, and invasion. MT1-MMP acts either through direct degradation of extracellular matrix components or indirectly by activating pro-MMP2 (55). In addition, these metalloproteinases are involved in the construction of the vascular tubular network, in part, through the release or the activation of growth factors (64, 65). TGFβ1, in turn, creates a favorable environment for tumor establishment by repressing immune surveillance, inducing the production of potent angiogenic factors such as vascular endothelial growth factor and basic fibroblast growth factor, and by increasing the production of extracellular matrix proteases (66, 67), which promotes tumor cell invasion and migration. In addition to TGFβ and MT1-MMP, the multiplicity of other established furin substrates involved in cell growth and survival (insulin-like growth factor receptor-1, platelet-derived growth factor, and hepatocyte growth factor) (8, 14, 68), cell invasion (E-cadherin and integrins) (3, 13), and angiogenesis (vascular endothelial growth factor-c) (69), support the contention that the regulation of furin activity within hypoxic/HIF-1-expressing zones of tumors could profoundly impact the course of tumor growth, invasion, and metastasis in a detrimental manner (Fig. 8).

The endopeptidase furin is considered to be the most important proprotein convertase of the constitutive secretory pathway, with an essential role in embryogenesis, homeostasis, and various diseases, through the proteolytic maturation of a diverse collection of proprotein substrates, including growth factors and their receptors. Among them, Insulin-like growth factor-1, hepatocyte growth factor, and platelet-derived growth factor have been shown to increase HIF-1α levels in various cell types (44, 70, 71). In addition, inflammatory cytokines such as tumor necrosis factor α, which is released from membranes by the furin substrate tumor necrosis factor α-converting enzyme/ADAM17 (72), induce HIF-1 activity in normoxic conditions (73, 74). These observations suggest that furin regulation by HIF-1 may be a more generalized phenomenon that could apply to other cellular contexts, including inflammatory condition, where furin activity was shown to be up-regulated (42).

REFERENCES


FIG. 8. Proposed impacts of furin hypoxia-induced expression. The proposed events depicted here are relative to tumorigenesis, but furin implication in other pathological processes is not excluded. Furin is known to maturate a broad range of growth factors, metalloproteinases, and adhesion molecules, some of the expression of which is increased upon hypoxia. Increased intracellular levels of furin in solid tumor hypoxic regions will impact the bioactivation of multiple tumorigenic/angiogenic factors and favor tumor progression.

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