Epigenetic Regulation of Gene Expression in Cervical Cancer Cells by the Tumor Microenvironment

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ABSTRACT

Evidence is accumulating that the adverse tumor microenvironment both modifies the malignant progression of tumor cells and contributes to chemotherapy and radiation resistance. We hypothesized that some of the effects on malignant progression are mediated through the transcriptional regulation of genes responsive to the stresses of the microenvironment, such as low oxygen or low glucose conditions. To determine epigenetic changes in gene expression that were consistent with that hypothesis, we used an in vitro subtractive hybridization method, representational difference analysis, to identify hypoxia-induced cDNAs from cultured human cervical epithelial cells. We identified 12 induced genes: two novel genes (HIG1 and HIG2), three genes known to be hypoxia-inducible (tissue factor, GAPDH, thioredoxin), and seven genes not previously identified as hypoxia-inducible [HNRNP(a1), ribosomal L7, annexin V, lipocortin 2, Ku(70), PRPP synthase, and acetoacetyl-CoA thiolute]. In cultured cells, HIG1 and HIG2 expression is induced by hypoxia and by glucose deprivation, but their expression is not induced by serum deprivation, UV, or ionizing radiation. The putative HIG1 and HIG2 open reading frames are expressed in cells, as confirmed by epitope tagging. In addition, tumor xenografts derived from human cervical cancer cells display increased expression of HIG1 and HIG2 when they are deprived of oxygen. Taken together, these data suggest a coordinated transcriptional response of eukaryotic cells to microenvironmental stresses found in the solid tumor.

INTRODUCTION

Tumor hypoxia is now being recognized as an independent prognostic indicator of poor patient survival in a number of tumor types (1–3), including squamous cell carcinoma of the uterine cervix (4). Interestingly, tumors with a low oxygen tension respond poorly to therapy regardless if they are treated by either chemotherapy or radiotherapy or even by surgery (4). Although radiation and some types of chemotherapy require oxygen to be maximally effective (5, 6), the fact that hypoxia predicted a worse outcome for the patients treated with surgery alone (4) implies that there is a fundamental biological difference in hypoxic tumors that cannot be explained by the effectiveness or access of the antitumor therapy. Additionally, model murine systems also identify a significant role for hypoxia-responsive genes in the growth of tumors in vivo (7, 8).

Hypoxia has also been shown to be a potent modulator of gene expression in a wide variety of cell lines tested in vitro. Specific protein accumulation (9, 10), gene induction (11), and gene repression (12) have been reported. Several transcription factors, AP-1 (13), NF-kB (14), and HIF-1α (15), have been identified whose activity increases under hypoxic conditions. HIF-1 is the transcription factor that responds most specifically and robustly to changes in oxygen concentration. This transcription factor is essential for development (8, 15) and is a heterodimer composed of a hypoxia-responsive HIF-1α subunit and a non-hypoxia responsive, constitutively expressed HIF-1β subunit (16). Under hypoxic conditions HIF-1α protein becomes stabilized (17) and binds with HIF-1β to an HRE containing the sequence 5’ACGTG(C/G)3’ (18). Although numerous HIF-1-responsive genes, such as VEGF (19), glycolytic enzymes (20), urokinase receptor (21), and endothelin I (22), have been reported in the literature, it is still unclear whether any or all of these gene products are the underlying reason why hypoxic tumors are more aggressive.

We hypothesize that determining the identity of hypoxia-induced genes would be critical for understanding the mechanism(s) responsible for the more aggressive nature of tumors that contain regions of hypoxia. In this communication, we describe the use of the RDA technique to identify hypoxia-induced sequence tags. Because the RDA technology has many potential technical pitfalls, the identity of each tag and the corresponding gene was confirmed by sequencing, and the hypoxia inducibility of each tag was confirmed by Northern blot.
ting. Using these rigorous criteria, the screen identified two novel expressed sequences that we cloned in their entirety; these two novel genes are termed HIG1 and HIG2. HIG1 and HIG2 may therefore represent a conserved mechanism for cells to respond to adverse microenvironmental stresses found within a tumor.

MATERIALS AND METHODS

Cell Lines and Tumor Formation. Normal human epithelial cells were immortalized in vitro by infection with retroviral constructs expressing HPV E6 and E7 oncoproteins. HCE.E6E7 were cultured in synthetic medium PFMR-4A (23). Normal human epithelial cells were immortalized in vitro by infection with retroviral constructs expressing HPV E6 and E7 oncoproteins. HCE.E6E7 were cultured in synthetic medium PFMR-4A (23).

RDA OLIGOS

| Linker 1 (DS) | TTTACCAAGCTTTATCCAAATTCGTCCTTCTCGACACAGGATGCGATG |
| Prim 1 (SS)  | ATGGTCAAAATAGTTAAGCACAGGAGGTGTCCCTCAC |
| Linker 2 (DS) | CCAGCTTTAACATTTGGTCGTAACAAAGCAGGAGATGCGATG |
| Prim 2 (SS)  | TATGGTCAAAATAGTTAAGCACAGGAGGTGTCCCTCAC |

HIG1

- hHIG1(for) AATTCTCTGACAGGAGGCGGGTGATGGA
- hHIG1(rev) GCCCTCGAAGGACATGTGTCACTAAAATTTAA
- mHIG1ds(for) CGGACTGAGAAGAAGACCCCGGTCGCTCGGA
- mHIG1ds(rev) GGCGCTCGAGTCTAGGCTGAATAGTCAGGACGTCATAAGGATAGCTAGGGCTTAGTTGCC

HIG2

- mHIG2(for) CCATTCTCTGACACAGGACAGTGG
- mHIG2(rev) GCCGCTGAGAAGAAGACCCCGGTCGCTCGGA
- hHIG2HA(for) CGTACAGGCTAAAAAGAGAGATGATGCGCTGTTCG
- hHIG2HA(rev) CGTACAGGCTAAAAAGAGAGATGATGCGCTGTTCG

RDA Technique. Briefly, the RDA technique (24) was performed on double-stranded cDNA that served as starting material for multiple rounds of in vitro subtraction and amplification. The cDNA was generated from mRNA isolated from control and 16-h hypoxia-treated HCE.E6E7 cells. The cDNA populations were digested with the restriction enzyme NlaIII and ligated to different double-stranded linkers described in Table 1 (linker 1 and linker 2). The modified cDNA fragments were then individually amplified using a single primer corresponding to the linker sequence described in Table 1 (primer 1 and primer 2). The primer amplifying the driver population of fragments contained a 5'-biotin label. Three micrograms of biotinylated, driver cDNA and 0.1 μg of tester, nonbiotinylated cDNA were mixed together, lyophilized, resuspended in 2 μl of hybridization buffer (50 mM HEPES, pH 7.5, 10 mM EDTA, 1.5 mM NaCl, and 2% SDS), covered in mineral oil, denatured at 95°C for 10 min, slowly cooled to 68°C over 1 h, and kept at 68°C for 4 more hours to allow hybridization. The hybridized cDNA populations were then diluted, mixed, and bound to 1 mg of M280 Dynal Streptavidin beads. The biotinylated DNA and any hybrid DNA was then removed with a magnet. The remaining, differentially expressed cDNA was reamplified using the tester primer, and then the subtraction was repeated three more times. The final cDNA population was digested with NlaIII and cloned into the Spfi site of pUC18 to generate the library of enriched fragments.

Northern Blotting and cDNA Isolation. Total RNA was isolated with TRIzol (Life Technologies, Inc., Grand Island, NY) following the directions of the manufacturer. Five to 10 μg of total RNA was denatured with glyoxal and size-fractionated on a 1% agarose phosphate gel. The gel was capillary-transferred to Hybond nylon (Schleicher and Shuell) and UV cross-linked. Probes were radiolabeled by random priming of gel-purified tag, full-length HIG1,4,*** or a fragment of HIG2

4 The HIG1 complete sequence can be found at NCBI GenBank accession no. AF145385 and HIG2 at no. AF144755.

\[ \text{[Equations]} \]
Table 2  Hypoxia-induced RDA tags

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<th>No. of hits</th>
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<th>Response</th>
<th>Comment</th>
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<td>HIG1</td>
<td>Novel</td>
</tr>
<tr>
<td>98</td>
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<td>HNRNP</td>
<td>HIG4</td>
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<td>Annexin V</td>
<td>HIG5</td>
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<td>HIG6</td>
<td></td>
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<tr>
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<td>not determined</td>
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<td>Lipocortin 2</td>
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*Minor 4.2-kb acetocetylCoA thiolase message is induced; response refers to mRNA changes by Northern blot analysis.

containing only the coding sequence in a Stul fragment (Rediprime; Amersham, Arlington Heights, IL). Hybridization was carried out in 0.5 M Na2HPO4, 7% SDS, 1 mM EDTA at 56°C for HIG1 and 65°C for HIG2, washed in 0.2–0.5× SSC at 56°C or 65°C, exposed to a phosphorimager plate, and visualized on a Storm 860 phosphorimager (Molecular Dynamics, Sunnyvale, CA). A CDNA library constructed from mRNA purified from SiHa cells exposed to 16-h hypoxia was used to isolate full-length HIG2. This library was probed with radiolabeled HIG2 tag using conventional methods. Full-length HIG1 was isolated by first identifying overlapping ESTs from the NCBI human EST database, until a full-length sequence was generated (1.35 kb). PCR primers were then synthesized corresponding 5’ and 3’ UTRs to amplify the complete sequence using RT-PCR of SiHa RNA isolated after a 16-h hypoxia treatment. The full-length HIG1 cDNA was then cloned and sequenced to confirm the predicted sequence. Recently, HIG1 has also been identified by another group as HSPC101, a gene expressed in hematopoietic stem cells (25).

**Construction of Epitope-Tagged HIG1 and HIG2.** HIG1-HA and HIG2-FA were constructed by reverse PCR. A (minus) primer was synthesized (Table 1, hHIG1HA[rev] or hHIG2HA[rev]) that hybridized to the carboxyl terminus of the ORFs. It extended the coding sequence by removing the enogenous stop codon, adding 36 nucleotides that code for 12 amino acids (the HA epitope), followed by a new stop codon, and ending with an MluI site. A second (plus) primer was synthesized (Table 1, hHIG1HA[for], or hHIG2HA[for]) that contained an MluI site, followed by a region that hybridized to the beginning of the 3’ untranslated region. The plasmid containing the cloned gene in an expression cassette (pEGFPN1 with the GFP removed; Clontech) was then used as a template for PCR amplification using pfu polymerase (Stratagene). The full-length linear molecule containing the added sequences was then digested with MluI, ligated closed, and used to transform competent bacteria.

**Immunological Detection of Epitope Tags.** For immunochemical detection of HIG1HA and HIG2HA, cells were grown on chamber slides and were transfected with the indicated constructs using LipofectAMINE according to the instructions of the manufacturer (Life Technologies, Inc.). After 48 h the transfected cells were fixed in 2% paraformaldehyde, washed two times with PBS-T, blocked for 1 h in PBS-T with 3% BSA, and incubated for 1 h with anti-HA monoclonal antibody (Babco 101R) at 1:500 dilution in PBS-T with 3% BSA. The anti-HA treated slides were washed three times with PBS-T, incubated with fluoresceinated secondary goat anti-mouse antibody (Vector), also in PBS-T-BSA, washed three times in PBS-T, caverslipped with antifade solution (Vector), and visualized under epifluorescence using a Nikon microphot fluorescent microscope.

For immunoblot detection of HIG1HA and HIG2HA, protein extracts were generated from cell populations transiently transfected with the indicated expression plasmids. Cell populations were harvested and resuspended in PBS containing the protease inhibitor PMSF, 1.0 mM of the phosphatase inhibitor Na3VO4, and 1.0 mM of the kinase inhibitor NaF. Twenty-five micrograms of the extracts were electrophoresed on a 15% tricine gel and electrotransferred to the polyvinylidine difluoride membrane. The membranes were then blocked with PBS-T containing 5% milk for >1 h, incubated with anti-HA antibody at a 1:2000 dilution for 1 h in PBS-T milk, washed three times in PBS-T, incubated with hors eradish peroxidase-conjugated goat antima mouse antibody at a 1:2500 dilution in PBS-T milk, washed three times in PBS-T, and visualized with enhanced ECL (Amersham, Rockford, IL) on the Storm 860 (Molecular Dynamics).
RESULTS

Human cervical epithelial cells stably immortalized with the HPV E6 and E7 oncoproteins served as the starting material for the construction of the RDA-enriched library. Four rounds of RDA subtraction of the oxic cDNAs from the hypoxic cDNAs generated a population of fragments representing genes that theoretically are induced by hypoxic treatment. Five hundred randomly chosen clones were partially sequenced, and these sequences were analyzed by NCBI BLAST to determine the frequency of each of the genes/ESTs in the enriched population. Because the most frequently repeated clones were unknown, we isolated full-length cDNAs that we decided to call HIG1 and HIG2. We then reanalyzed the remaining unknown fragments from our 500 sequences against these complete genes, so that we could categorize all of the hypoxia-induced tags with respect to the two new unknown genes. The subtraction of hypoxic mRNAs from oxic mRNAs, designed to identify hypoxia-repressed genes, was not performed because hypoxia globally reduces transcription and so it is more difficult to identify genes that are specifically repressed.

Because some of the genes are represented multiple times in this library, the 500 tags represent fragments of 21 genes that are present more than one time and 168 genes that are represented only once (Table 2). The two most frequently occurring genes are HIG1 and HIG2. All of the clones represented more than one time that did not contain a highly repetitive element were tested by Northern blot for induction by hypoxia as well as by hypoxia and reoxygenation in SiHa cervical carcinoma cells. Representative Northern blot analysis is shown in Fig. 1 to demonstrate the kinetics of induction for each of the induced genes. It is interesting to note that approximately one third of the tags in Table 2 (7/19) were not induced, so it becomes clear why it is necessary to test each probe by Northern analysis. Although acetoacetyl-CoA thiolase sequence tag is listed as induced, the reported, major RNA (1.8 kb) for the gene does not change. However, there is a larger, hybridizing RNA species (4.2 kb) that is induced after 24–48-h hypoxia (data not shown). The Northern blot for annexin V is shown from HCE.E6E7 cells because annexin V is weakly induced in SiHa cells.

There are clearly distinct patterns of induction by hypoxia: one group of genes is induced to moderate levels with early kinetics (within 2–6 h), followed by a diminution (after 12 h), whereas another group of genes is induced to higher levels, but only after prolonged exposure to hypoxia (12–24 h). Interestingly, those genes that are induced by short-term hypoxia also seem to be induced by reoxygenation, [e.g., HIG1, HNRNP(A1), Ku(70), and thioredoxin]. The genes listed in Table 1 that are not shown in Fig. 1 had only moderate hypoxic induction (2–3-fold). There are three genes identified in this group that have been previously reported to be hypoxia-inducible: GAPDH (26), tissue factor (27), and thioredoxin (28).

Because HIG1 and HIG2 represent two novel genes whose functions are unknown, we investigated these genes in more detail. We first examined the expression of HIG1 and HIG2 in a series of cervical cancer cell lines under oxic and hypoxic conditions (Fig. 2). Although HIG1 is induced moderately within 2 h of hypoxia in all of the cell lines tested, it only remains elevated only in the SiHa cells. HIG2 is more consistently induced from low basal levels in all of the cervical cancer cells tested. The major HIG2 mRNA species is 1.4 kb in length, but there are two other mRNA species of minor abundance (8.0 and 9.0 kb) that are induced with identical kinetics to the major species (data not shown). The hypoxic induction of HIG1 and HIG2 in vivo was also tested in tumor xenografts generated from the C33a cell line by Northern blot analysis of total tumor RNA. We compared untreated xenografts to xenografts that were made hypoxic by treatment of the host animal with FAA 24 h prior to explantation and RNA isolation (Fig. 2). We chose to examine expression changes after 24 h because this is the time shown to result in the most dramatic ablation of the tumor vasculature before causing extensive parenchymal necrosis. FAA treatment resulted in increased tumor hypoxia as measured by Eppendorf electrode (data not shown) and increased HIG1 and HIG2 expression by 1.2- and 2.4-fold, respectively. The moderate level of HIG1 induction in vivo is not unexpected, because of the in vitro data. The portion of the human gene used as a probe in these experiments has low homology with mouse RNA and under the conditions used did not cross-hybridize.

The translated product of the putative ORFs from both genes is shown in Fig. 3. Both ORFs encode small peptides (93 to 188 amino acids). The translated product of the putative ORF from HIG1 shows high homology to HNRNP(A1) and E6AP (Table 3), whereas the translated product of the putative ORF from HIG2 is most similar to the KIAA0079 gene [GenBank: U37881]. The putative ORFs of both genes were therefore named Ku(70) and HNRNP(A1), respectively.
and 63 amino acid residues). Both peptide sequences were run through NCBI pBLAST, and no existing protein sequences of significant similarity were found. No functional motifs were identified in either sequence using world wide web-based search programs Prodom-Blast at Institut National de la Recherche Agronomique5 or Propsearch at European Molecular Biology Laboratory-Heidelberg. 6

We next investigated whether HIG1 and HIG2 induction is unique to hypoxic stress or if it is elicited by other tumor microenvironment stresses, such as glucose deprivation or serum starvation, or by genotoxic stresses, such as UV or ionizing radiation. We also tested the hypoxia-mimetic stress inducer deferroxamine, as well as glucose deprivation. UV light seemed to have little effect upon either HIG1 or HIG2 expression. In contrast, although ionizing radiation did not change HIG1 expression levels, it did result in a moderate 2.5-fold induction of HIG2 by 24 h. The similarities in the pattern of stress responsiveness of HIG2 and that of the HIF-responsive VEGF gene suggest that HIF-1 may be important in HIG2 expression.

Finally, we determined if the ORFs that were identified in HIG1 and HIG2 were actually translated in vivo. To determine this, HA epitopes were added to the 3' end of the putative ORF of both HIG1 and HIG2 by PCR, and the chimeras were expressed off of the CMV immediate early promoter after transient transfection into C33a cells. After 48 h, cells transfected with either CMVHIG1HA or CMVHIG2HA were fixed and incubated with anti-HA antibody to determine intracellular localization of the expressed product (Fig. 5C, HIG1; Fig. 5F, HIG2). Extracts of these cells were also examined by immunoblot for the protein size (Fig. 5A, HIG1,5D, HIG2). Expression of a peptide is detected only in the cells transfected with plasmids expressing the tagged protein(s).

Immunoreactive material was detected in punctate pattern throughout the cytoplasm for HIG1, suggesting a vesicular or mitochondrial location. Immunoreactive material was found in a more diffuse, cytoplasmic localization for HIG2. It is difficult to draw too many conclusions from the cellular localization patterns of proteins that are overexpressed. Forced overexpression of some proteins can overwhelm normal trafficking patterns leading to aberrant localizations. Cellular morphology can be delineated by viewing Fig. 5, B and D, which represent the same fields seen in Fig. 5, C and F, but visualized under UV to excite the DAPI-stained nuclei. Because HIG2 appeared on the immu-

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6 Address: http://www.embl-heidelberg.de/prs.html.
As multiple bands, we hypothesize that this might represent multiple processed forms of the protein, producing altered migration. Interestingly, hypoxic treatment did not alter the protein quantity or electrophoretic mobility of the heterologously expressed proteins in C33a cell populations transfected with CMVHIG1HA or CMVHIG2HA and treated with hypoxia for 6 h (Fig. 5, A and D).

**DISCUSSION**

RDA analysis of HPV-immortalized cervical cells led to the identification of hypoxia-induced genes of several functional classes: genes involved in DNA metabolism, intermediate cellular metabolism, tissue structure, and angiogenesis. Two of the genes, *HIG1* and *HIG2*, are novel and have unknown function; three of the genes have already been reported to be hypoxia-inducible: *GAPDH* (26), *tissue factor* (27), and *THX* (28); and six are known genes that had not previously been shown to be hypoxia-inducible: *HNRNPA1*, ribosomal protein L7, annexin V, lipocortin 2, *PRPP synthase*, and acetocacetyl-CoA thiolase. It is not yet clear if these genes are responsible for the more aggressive nature of hypoxic tumors, but *HIG1* and *HIG2* are shown here to increase in expression in tumor xenografts when they become more hypoxic (treatment with FAA). At present we can only speculate as to the function of these genes in the hypoxic tumor. Examining the regulation of expression of genes such as *HIG1* and *HIG2* in response to microenvironmental stresses yields insight into the epigenetic regulation of the hypoxic tumor phenotype. Coordinated expression of genes in

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**Fig. 4** Stress inducibility of *HIG1* and *HIG2* in C33a cells. Northern blot analysis of RNA isolated from C33a cells that were treated with hypoxia, glucose deprivation (0.0 mM glucose), serum deprivation (0.1%), UV light (20 J/m²), or ionizing radiation (8 Gy). RNA was isolated at 6 and 24 h following treatments. The same membrane was probed sequentially with *HIG1* and *HIG2*; methylene blue stain of 18S rRNA for loading control.

**Fig. 5** Identification of immunoreactive material from epitope-tagged expression of HIG1HA (A–C) and HIG2HA (D–F). Immunoblot (A, D) of whole cell extracts from C33a cells transiently transfected with the indicated expression plasmids and probed with anti-HA monoclonal antibody (Baco 101R). Lanes labeled (h) indicate 6-h hypoxia treatment. Immunofluorescence of transfected cell population that was first fixed and then incubated with anti-HA antibody, and visualized with fluorescent antiserum. A, HIG1HA cells visualized with DAPI DNA stain; B, HIG1HA cells visualized with DAPI DNA stain; C, same field visualized with anti-HA antibody; D, HIG2HA cells visualized with DAPI stain; E, same field visualized with anti-HA antibody.
response to multiple microenvironmental stresses, such as hypoxia or glucose deprivation, leads to a cell that could be resistant to further stresses.

The HIF-1α gene product has been shown to participate in the repair of DNA double-strand breaks and could hypothetically respond to hypoxia-induced DNA damage (29) if it occurs. Another possibility is that hypoxia-stimulated HIF-1α expression could act as a tumor suppressor (30) as has been reported. Regardless of the reason, if low oxygen leads to HIF-1α induction, one side effect could be increased resistance to DNA-damaging agents or ionizing radiation.

Hypoxia has been shown to induce the expression of rate-limiting enzymes necessary for increased glycolysis in the absence of oxidative phosphorylation (20). One additional product of glycolysis is the generation of (reduced) NADH. The major generation of NADH during glycolysis is from the activity of GAPDH. The induction of GAPDH therefore serves two functions: (1) to contribute to increased glycolysis, and (2) to increase the production of reduced NADH. If hypoxic damage were mediated through a redox imbalance, then it would also be reasonable to induce a system for titrating the extra reducing/oxidizing equivalents. These extra reducing equivalents also could be used by several different cellular processes. For instance, it has been shown that thiolredox can use NADH as a proton donor to activate the transcription factor AP-1 (31) or the estrogen receptor (32). To generate a large cellular pool of NADH, it might be necessary to synthesize more NAD. One of the precursors for NAD is adenine, and the rate-limiting step in the de novo generation of purines is PRPP synthase. Thus, PRPP synthase induction by hypoxia may be a physiological response to a redox imbalance.

Annexin V and lipocortin 2 code for two family members of a group of cell surface calcium-binding proteins. Both molecules have been shown to play a role in the regulation of the fibrinolytic activity of plasmin (33). These gene products also decrease cell motility in vitro (34). Thus, as the cell surface properties of hypoxic cells could regulate adhesion and cell-cell connections in a tumor, the annexins, in concert with hypoxia-responsive proteases such as calpain (35), could impact tumor invasiveness and metastatic potential.

Wound healing, clot formation, and revascularization rely on delicately balanced factors, and one of the most potent inducers of angiogenesis is tissue hypoxia. It is not clear what the signaling mechanism is that leads to vessel generation, but many hypoxia-responsive genes are involved, such as VEGF (19) and endothelin 1 (22). Hypothetically, hypoxia could act as a regulator of fibrinolysis by modification of plasmin activity through annexin induction (33). Additionally, tissue factor has been shown to regulate both the generation of fibrin (36) and the angiogenic activity of VEGF (37). Thus, tissue factor expression in response to hypoxia could be important in regulating the function of the vasculogenic factors of the tumor and as such could influence the growth rate of the tumor.

There are several reasons why we did not isolate all of the known hypoxia-inducible genes (such as VEGF) in this series of experiments. The primary reason is the nonrandom distribution of cleavage sites for the restriction enzyme NlaIII used for digestion of cDNAs prior to linker ligation. Those genes, such as VEGF, with NlaIII restriction sites organized in a nonrandom manner would generate fragments outside the 100- to 300-bp size that is most efficiently hybridized with the current protocol. The unlinked fragments, or the very large or very small fragments would be lost through the multiple rounds of subtraction and amplification. Another possibility is that additional bona fide hypoxia-inducible genes exist in the list of single hits from the 500 sequences. We chose to stop sequencing clones at 500 because we feel that this number gives good coverage to the complexity of our RDA library. We make this statement after comparing the diversity of the first 100 clones that were sequenced to the diversity of the next 400 clones that were sequenced.

Human HIG2 has a high fraction of serine and threonine residues, 8 and 6 residues, respectively, of the total of 64 amino acid residues (Fig. 3). Serine 41 conforms to the consensus PKC recognition motif and is conserved in rodents and humans and consists of the predicted size. Thus, PRPP synthase induction by hypoxia may be a physiological response to a redox imbalance.

Taken together, these data support the hypothesis that there exists a cellular response to hypoxic stress that is regulated at the transcriptional level. This response is evolutionarily conserved in rodents and humans and consists of the coordinated regulation of many genes by HIF-1-dependent mechanisms as well as HIF-1-independent mechanisms so that the cell can survive in this adverse environment. The byproducts of this epigenetic response to low oxygen results in a tumor that is more resistant to conventional therapy and is more likely to invade or metastasize. The genes described in this report therefore represent potential new hypoxia-regulated proteins that can influence clinical outcome.

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