Hypoxic Regulation of Id-1 and Activation of the Unfolded Protein Response Are Aberrant in Neuroblastoma*

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The Id proteins play an important role in proliferation, differentiation and tumorigenesis. Many tumors are hypoxic, but it is unknown if expression of Id proteins is regulated in hypoxic cells. Here we show that Id-1 is down-regulated in multiple primary, immortalized, and neoplastic hypoxic cell lines, and the transcriptional repressor ATF-3 is both necessary and sufficient for this hypoxia-induced repression of Id-1. Hypoxic up-regulation of ATF-3 is due in part to activation of the unfolded protein response, a cellular stress response. Remarkably, we observe that the unfolded protein response is de-regulated in all neuroblastoma cell lines tested. Indeed, in the absence of ATF-3 the hypoxia-induced transcription factor HIF-1 α-regulates Id-1 in hypoxic neuroblastoma cells. Hypoxic neuroblastoma cells diminish expression of some neuronal differentiation markers, and forced expression of ATF-3 in hypoxic neuroblastoma cells represses Id-1 and prevents the loss of these markers. The divergent regulation of Id proteins in distinct hypoxic cells may explain some of the varied effects hypoxia has on cellular differentiation and proliferation.

Many tumors are profoundly hypoxic and multiple studies have demonstrated that hypoxic tumors have a poorer prognosis than non-hypoxic tumors. Although the full etiology of this observation is unclear, many of the phenotypes associated with hypoxic cells are due to the induction and suppression of gene expression (reviewed in Ref. 1). While the hypoxia-inducible transcription factor, HIF-1, is the best characterized inducer of gene transcription in hypoxic cells, it is clear that additional signaling pathways can both up-regulate and down-regulate gene expression in hypoxic cells (2, 3). In addition, HIF-1 targets differ dramatically in various cell types despite similar expression of HIF-1α (3–6) suggesting that HIF-1 transcriptional activity may be modulated by cell specific factors.

The existence of cell-specific factors may play a role in the marked phenotypic differences noted between different hypoxic cell lines and tumors. For example, most normal cells and many neoplastic cells undergo a growth arrest when hypoxic, whereas some stem cells and neoplastic cells continue to proliferate under hypoxic conditions (7, 8). Studies have also suggested that hemangioblasts, renal tubular cells, and embryonic stem cells all differentiate when hypoxic, while hypoxic adipocytes and hematopoietic stem cells are resistant to differentiation (9–12). Neuroblastoma cells, when rendered hypoxic, lose some neuronal markers, leading to the hypothesis that they undergo hypoxic “de-differentiation” to immature neural crest cells (13). Despite the probability that hypoxic regulation of proliferation and differentiation play an important role in the aggressiveness of hypoxic tumors, the mechanisms by which hypoxic cells regulate proliferation and differentiation have not been fully delineated.

The Id proteins play an important role in both proliferation and differentiation, although their regulation in hypoxic cells has not been well studied. Id proteins, consisting of Id-1, Id-2, Id-3, and Id-4, act as inhibitors of DNA binding and inhibitors of differentiation. These proteins contain helix-loop-helix (HLH) motifs, but unlike HLH transcription factors they do not contain DNA binding domains. Thus Id proteins can heterodimerize with HLH transcription factors, prevent these transcription factors from binding to DNA and transactivating genes, and act in a dominant negative fashion. Id proteins can heterodimerize with two classes of HLH transcription factors. Class I HLH transcription factors are ubiquitous and encompass the E2A transcription factor, which functions as a tumor suppressor and inhibits proliferation. Class II HLH transcription factors are tissue-specific and regulate genes necessary for nerve, muscle, and T cell development (reviewed in Ref. 14).

The importance of Id family members in cancer is supported by the fact that Id overexpression promotes immortalization (15), proliferation (16), and angiogenesis and neo-vascularization (17, 18). The ability of Id proteins to inhibit differentiation may play an important role in tumorigenesis, as loss of differentiation is a hallmark of cancer cells. The Id proteins play a particularly well documented role in the biology of neuroblastoma. Id family members are highly expressed in neuroblastoma, prevent pharmacological differentiation of neuroblastoma cells lines (19, 20), and may indicate a poor prognosis (16, 19).

Through a non-biased screen for transcription factors altered in hypoxic fibroblasts we determined that Id-1 expres-
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sion is decreased in a variety of primary, immortalized, and neoplastic cells when they are rendered hypoxic. We therefore pursued the mechanism and significance of hypoxic Id-1 regulation and sought to determine whether hypoxic regulation of Id-1 is aberrant in some cancers.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—ATF-3−/− MEFs and Corresponding Wild-type MEFs were the kind gift of Twosnin Hai (21), ATF-4−/− MEFs (22) and PERK−/− MEFs (23), as well as their corresponding wild-type MEFs, were the generous gift of David Ron, and eukaryotic initiation factor 2 (eIF2α) S51A knock-in MEFs and corresponding wild-type MEFs were the gift of R. Kaufman (24). Neuroblastoma cell lines were obtained from S. Lowe) using PCR primers upstream (US) 5′-CGAATGGGACGTCAGGCCTGAGGC and 3′-GGACACGGACAGGATTCACTGGTTCCGACTTCAGTGCCGCAGCCGCTGCAGGC and downstream (DS) 5′-ATGTCGAGCGTCATCTGTGATGCGCAGAGCATCTGTGACCCCATCTGGAATTGAGATGCTGACGAGCAG-3′ (wild-type) or 5′-GGAGCCATCATGTCGAGTCAGGCAGGAC-3′ (ΔD2) and 3′-GGAATGGGAAACAGAGTAGCAGCTTCTCTCGTTAGTGTCAG-3′. Retrovirus was obtained by co-transfecting 293GP cells (gift of Frances Ventura, was transfected into human kidney epithelial cells using FuGENE 6. After 24 h or recovery cells were rendered either hypoxic or maintained as normoxic for an additional 16 h, at which time luciferase activity was determined with the Promega luciferase assay system according to the manufacturer’s protocol.

Electromobility Shift Assay (EMSA)—EMSA were performed as described (8). Oligonucleotides for the Id-1 promoter/luciferase constructs (26), the kind gift of Frances Ventura, were transfected into human kidney epithelial cells using FuGENE 6. After 24 h or recovery cells were rendered either hypoxic or maintained as normoxic for an additional 16 h, at which time luciferase activity was determined with the Promega luciferase assay system according to the manufacturer’s protocol.

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RESULTS

Id-1 Is Down-regulated in Hypoxic Cells via Transcriptional Repression Mediated by ATF-3—During the course of an unbiased screen to identify transcription factors with altered activity in hypoxic cells, we determined that Id-1 protein expression is diminished in a variety of primary, immortalized, and transformed cell lines (Fig. 1A and data not shown). Because down-regulation of Id-1 might explain in part why some hypoxic cells undergo a growth arrest and/or differentiate, we pursued the mechanism for Id-1 down-regulation in hypoxic cells.

Previous studies have demonstrated that Id-1 expression can be regulated at both the post-translational and transcriptional level (27, 28). To delineate the mechanism for decreased Id-1 expression in hypoxic cells we first investigated potential post-translational regulation of Id-1 in hypoxic cells. Mouse embryo fibroblasts (MEFs) stably expressing tagged Id-1 from a retroviral construct were treated with cycloheximide, and lysates were collected at regular intervals and subjected to immunoblot to assess stability of the Id-1 protein. There was no significant difference in the half-life of endogenous versus exogenous Id-1 proteins in normoxic (N) or after cells had been rendered hypoxic (Hy) for 16 h. Id-1 expression was determined by anti-Id-1 or anti-FLAG immunoblot for the endogenous and exogenous proteins respectively. Densitometry of bands from three to four independent experiments was determined, and the average ± S.E. are displayed graphically below the immunoblots.

In contrast to endogenous Id-1 protein expression, exogenous Id-1 expression (where Id-1 transcription is mediated by a retroviral LTR promoter instead of the native Id-1 promoter) was not repressed in hypoxic cells (Fig. 1B) suggesting that endogenous Id-1 is regulated at the transcriptional level in hypoxic cells. In addition, the half-life of exogenous Id-1 protein was not significantly altered in hypoxic cells compared with normoxic cells (Fig. 1B, with the average of three independent experiments ± S.E. shown graphically below). Together, these data demonstrate that Id-1 translation and degradation are not altered in hypoxic cells and suggest Id-1 may be transcriptionally repressed in hypoxic cells.

We next examined directly whether transcriptional repression of the Id-1 promoter occurs in hypoxic cells. MEFs rendered hypoxic demonstrated a rapid decrease in Id-1 mRNA as determined by semiquantitative RT-PCR and quantitative PCR (Fig. 1C and D). To determine whether the decrease of Id-1 mRNA in hypoxic cells is due to direct transcriptional repression of the Id-1 promoter or a result of decrease in Id-1 mRNA stability, we transfected a luciferase construct containing −1.4 kilobases of the Id-1 proximal promoter (26) into human kidney epithelial cells and rendered these cells hypoxic for 16 h. When compared with transfected normal cells, there was a significant repression of the Id-1 promoter-driven reporter in hypoxic cells (Fig. 1E). In total, these data demonstrate that transcription of Id-1 is repressed in hypoxic cells.

FIGURE 1. Id-1 is down-regulated in hypoxic cells by ATF-3. A, a variety of cell lines were rendered hypoxic for 16 h, and Id-1 protein levels were determined by anti-Id-1 immunoblot. Coomassie Blue staining of duplicate gels (coom.) serves as a loading control. B, MEF cells were stably infected with vectors expressing either Id-1 or empty vector controls, and cell lysates were collected after the indicated periods of cycloheximide (CHX) treatment in normoxic (N) or after cells had been rendered hypoxic (Hy) for 16 h. Id-1 expression was determined by anti-Id-1 or anti-FLAG immunoblot for the endogenous and exogenous proteins respectively. Densitometry of bands from three to four independent experiments was determined, and the average ± S.E. are displayed graphically below the immunoblots.

C, Id-1 RNA is diminished in 16 h hypoxic wild-type MEFs but not in hypoxic ATF-3 knock-out MEFs as determined by semiquantitative RT-PCR (above, with actin RT-PCR serving as a control for input RNA integrity) and by quantitative real-time PCR (D). *, p = 0.03 by two-sided Student’s t-test. E, human kidney epithelial cells were transfected with Id-1 promoter/luciferase reporters (above) as detailed under “Experimental Procedures.” After 16 h luciferase activity was assessed; data reflect hypoxic/normoxic luciferase activity of the Id-1 promoter and the Id-1 promoter with a mutated ATF motif, normalized to empty pGL2 vector. F and G, immunoblots reveal that ATF-3 is induced in a variety of hypoxic (Hy) primary, immortalized, and transformed cell lines including HaCAT (C) and MEF and U2OS cells (D) when compared with normoxic (N) cells. Coomassie (coom.) serves as the loading control.
We then pursued the mechanism by which Id-1 is transcriptionally repressed in hypoxic cells. TGFβ-1 has recently been reported to down-regulate Id-1 mRNA in immortalized HaCAT (human keratinocytes) cells via an ATF-3 binding site in the Id-1 proximal promoter (27). ATF-3 is a member of a family of stress-induced transcription factors. Whereas other ATF family members are transcriptional activators, ATF-3 has been reported to serve either as a transcriptional activator or repressor, depending on the cellular context (reviewed in Ref. 29). We found that HaCAT cells also down-regulate Id-1 and up-regulate ATF-3 when rendered hypoxic (Fig. 1F), as do a number of other normal and transformed cell lines (Fig. 1G and data not shown). Quantitative RT-PCR confirmed a greater than 5-fold reduction of Id-1 mRNA and 5-fold up-regulation of ATF-3 mRNA in wild-type MEFs by 4 h (Fig. 1D). Importantly Id-1 RNA is not repressed in hypoxic ATF-3−/− MEFs, and in fact a modest but significant up-regulation of Id-1 mRNA was noted in hypoxic ATF-3−/− MEFs at 8 h (Fig. 1D).

Similarly, Id-1 protein expression was only mildly repressed in hypoxic ATF-3−/− MEFs as compared with wild-type MEFs (data not shown). In contrast to the native Id-1 proximal promoter, an Id-1 promoter construct with a mutated ATF site was not repressed in hypoxic cells (Fig. 1E). Thus hypoxia-induced ATF-3 is necessary for the hypoxic repression of Id-1.

**ATF-3 Is Induced in Hypoxic Cells in Part by the Unfolded Protein Response**—Since our data demonstrate that ATF-3 is necessary for the hypoxia-induced down-regulation of Id-1, we next focused on the mechanism of the hypoxic up-regulation of ATF-3. ATF-3 is induced by cytokines and a variety of stresses, including UV irradiation, DNA damage, and ischemia/reperfusion (reviewed in Ref. 29). The transcriptional activator ATF-4 has also been implicated in the up-regulation of ATF-3 (23). ATF-4, in turn, can be induced by multiple stresses including protein misfolding in the endoplasmic reticulum and subsequent activation of the unfolded protein response (UPR). The UPR is a stress response pathway in which a global inhibition of protein synthesis leads to increased expression of stress response genes which serve to adapt cells to this increased stress. One of the central mechanisms in this stress response is the phosphorylation of the alpha subunit of eIF2α on serine 51 by the UPR-activated PERK kinase (30). Phosphorylation of eIF2α by the PERK kinase leads to both general translational inhibition and a paradoxical increased translation of a small subset of proteins, including ATF-4. Because both activation of the UPR and up-regulation of ATF-4 have recently been demonstrated to occur in hypoxic cells (31–34), we explored whether hypoxic activation of the UPR and up-regulation of ATF-4 leads to the hypoxic up-regulation of ATF-3 and subsequent repression of Id-1.

When compared with wild-type MEFs, ATF-3 induction was diminished in hypoxic ATF-4−/− MEFs (Fig. 2A) and PERK−/− MEFs (Fig. 2B). In hypoxic MEFs in which the endogenous eIF2α gene has been genetically replaced by an eIF2α allele, which cannot be phosphorylated (S51A) (and thus the PERK dependent branch of the UPR cannot be activated) (24), ATF-4 expression was entirely blunted, and both ATF-3 induction and Id-1 repression were diminished 3-fold (Fig. 2C, quantitation in right panel). Consistent with hypoxic activation of the unfolded protein response, ATF-4 mRNA was not increased in hypoxic cells suggesting that the increased expression of ATF-4 is due to translational regulation (Fig. 2D). Also consistent with activation of the UPR pathway, ATF-3 and an additional ATF-4 target CHOP were induced at the mRNA level in hypoxic cells (Fig. 2D). Thus we conclude that in hypoxic cells PERK is activated, leading to eIF2α phosphorylation and ATF-4 generation, which in turn leads to ATF-3 induction and Id-1 repression (Fig. 2E). Because some detectable ATF-3 is generated in hypoxic PERK−/−, ATF-4−/−, and eIF2α S51A cells, there are additional mechanisms for the hypoxic induction of ATF-3 (dashed lines in Fig. 2E). Other signaling pathways implicated in the induction of ATF-3 include the p38/MAP kinase signaling pathway (reviewed in Ref. 29); however, we did not note either activation of p38 in hypoxic MEFs or alteration of ATF-3 expression with p38 inhibition in hypoxic MEFs (data not shown). We have, however, noted significant stabilization of ATF-3 mRNA in hypoxic cells.3 What role this prolongation of ATF-3 mRNA half-life has in explaining UPR independent mechanisms for ATF-3 induction and Id-1 repression in hypoxic cells remains to be determined.

The Induction of ATF-3 and Repression of Id-1 Does Not Occur in Neuroblastoma Cell Lines—Because Id-1 is highly expressed in many cancers and appears to play an important role in tumorigenesis, we hypothesized that hypoxia-induced down-regulation of Id-1 might be aberrant in some neoplastic cells. Although we observed that Id-1 protein is down-regulated in over 10 cell lines, including several neural and astrocytoma cell lines, when rendered hypoxic (data not shown), previous literature suggested that Id-1 and Id-2 mRNA are actually induced in several hypoxic neuroblastoma cell lines at short time points (19). Under our conditions, we also observed that indeed Id-1 protein was induced after 16 h of hypoxia in multiple neuroblastoma cell lines (SK-N-BE(2), SH-SY5Y, and SK-N-SH) (Fig. 3A). To determine why the UPR does not down-regulate Id-1 in hypoxic neuroblastoma cells as it does in the many other cell lines we studied, we assessed the activation of the UPR in neuroblastoma. Remarkably, ATF-3 was minimally induced in hypoxic neuroblastoma cells at the protein or RNA level (Fig. 3A). In contrast to U2OS cells, ATF-3 was also not expressed in neuroblastoma cells with other ER stresses, such as tunicamycin, thapsigargin (data not shown), or the calcium ionophore A23187 (35) (Fig. 3B).

ATF-4 was also not induced in neuroblastoma cells rendered hypoxic for 4, 8, or 16 h (data not shown and Fig. 3C). Induction of CHOP, a pro-apoptotic protein that is another ATF-4 target, was also dependent on eIF2α phosphorylation and was also not observed in hypoxic neuroblastoma cells (Fig. 3C). Since the central component of the UPR is eIF2α phosphorylation, we examined the phosphorylation status of eIF2α in hypoxic neuroblastoma cells. There was no difference in total amount of eIF2α in neuroblastoma cells when rendered hypoxic (Fig. 3C). However, we noted that as opposed to the marked phosphorylation of eIF2α in hypoxic MEFs, there was a paradoxical high basal level of eIF2α phosphorylation in normoxic neuroblas-

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3 L. B. Gardner, unpublished data.
toma cells, which did not markedly increase when these cells were rendered hypoxic (Fig. 3C).

The lack of ATF-4 up-regulation and the induction of ATF-4 targets in hypoxic neuroblastoma cells could be due to a specific defect in the PERK-eIF2α axis or alternatively due to a general reduction in ER stress (i.e. misfolded proteins) either generated or sensed in hypoxic neuroblastoma cells. To evaluate these potential explanations, we explored the activation of a PERK-independent branch of the UPR in neuroblastoma cells, splicing of the mRNA for the transcription factor XBP1, which is mediated by IRE1 endonuclease activation by ER stress (36). When neuroblastoma cells were either rendered hypoxic or treated with a chemical inducer of ER stress, there was significant splicing of XBP1, comparable with the splicing seen in HaCAT cells or the glioblastoma cell line LN229 (Fig. 3D), demonstrating that ER stress is generated in neuroblastoma cells, and the UPR defect in these cells is specifically in the PERK-eIF2α-dependent branch of the UPR.

Finally, to determine whether the lack of UPR activation (and subsequent lack of ATF-3 induction) in hypoxic neuroblastoma cells was necessary for Id-1 induction, we created a retrovirus for exogenous expression of ATF-3. As seen previously, ATF-3 was up-regulated and Id-1 was down-regulated in hypoxic HaCAT cells but not in hypoxic SK-N-BE(2) cells (Fig. 3E). Importantly, when ATF-3 was constitutively expressed in neuroblastoma cells, Id-1 induction was prevented when cells were rendered hypoxic (Fig. 3E). These data demonstrate a causal role of ATF-3 induction and the UPR in the hypoxic repression of Id-1.

HIF-1 Transactivates Id-1 in the Absence of the UPR—Although the lack of ATF-3 up-regulation in hypoxic neuroblastoma cells explains why Id-1 expression is not repressed in
these cells, we noted an actual induction of Id-1 in hypoxic neuroblastoma cells (Fig. 3, A and E). A similar, although more modest, induction of Id-1 was noted in hypoxic ATF-3−/− MEFS (Fig. 1F). Because Id-2 has been demonstrated to be a HIF-1 target in neuroblastoma cells (37), we sought to determine whether Id-1 is also a HIF-1 target. We constructed a lentivirus expressing a shRNA directed against HIF-1α. Expression of this shRNA efficiently knocked down HIF-1α expression in hypoxic SK-N-BE(2) cells as compared with a scrambled sequence (Fig. 4A). In the absence of HIF-1α, hypoxic neuroblastoma cells did not up-regulate Id-1 or the HIF-1 target VEGF (Fig. 4A). In addition, knock-down of HIF-1α, which resulted in a reduction of hypoxic mRNA up-regulation of the HIF-1 targets enolase and lactate dehydrogenase, also resulted in a 10-fold reduction in Id-1 mRNA induction in neuroblastoma cells (Fig. 4B). Therefore, hypoxic cells may either repress Id-1 though the generation of ATF-3, or up-regulate Id-1 through HIF-1, as seen in neuroblastoma cells.

Since these data demonstrate that Id-1 is regulated by HIF-1 in hypoxic neuroblastoma cells, we sought to determine whether Id-1 is a direct HIF-1 target. The ATF (CREB) binding site responsible for TGFβ-induced, ATF-3 mediated, repression of Id-1 (27) contains an evolutionarily conserved overlapping putative HIF-1 binding motif as determined by a computer-generated algorithm. Therefore we hypothesized that ATF-3 generation in hypoxic cells interferes with HIF-1 transactivation of the Id-1 promoter; this antagonism of HIF-1 and ATF-3 is not expected to occur in hypoxic neuroblastoma cells where ATF-3 is not up-regulated. We used the region of the Id-1 promoter that contains the HIF-1/ATF binding sites as a probe to perform EMSAs on hypoxic neuroblastoma cells, which induce Id-1 but not ATF-3 (Fig. 3A), and hypoxic MEF and HaCAT cells, which induce ATF-3 and repress Id-1 expression (Fig. 3E).

In hypoxic SK-N-BE(2) cells a complex formed that could be selectively shifted with a HIF-1α antibody (Fig. 4C, lanes 2 and 9). A similar complex was seen in another hypoxic neuroblastoma line, SH-SY5Y (data not shown). Wild-type oligonucleotide competed for the hypoxia increased HIF-1 complex (lanes 3 and 4), although full competition required 30-fold cold oligonucleotide (data not shown). Intriguingly, an oligonucleotide mutated at the ATF site competed more effectively for this HIF-1 complex than wild-type oligonucleotide (lanes 5 and 6 versus lanes 3 and 4).

In addition, when this ATF-mutated oligonucleotide was used as a probe it bound HIF-1 as well or better than wild-type oligonucleotide (lanes 7 and 8 versus lanes 1 and 2). These data suggest that HIF-1 directly binds to the Id-1 promoter and this binding is increased in the absence of ATF binding.

This HIF-1 complex was not noted in MEF and HaCAT cells, despite equivalent induction of HIF-1α protein expression (data not shown). In MEF and HaCAT cells we noted a less intense, slower migrating hypoxia-induced complex that competed with wild-type oligonucleotide (lanes 15 and 16) but not with an oligonucleotide with the ATF-3 site mutated (lanes 17 and 18). This slower migrating complex was not present in the neuroblastoma cells. In contrast with the HIF-1 complex, this complex did not form when the mutated ATF-3 oligonucleotide was used as a probe (lanes 19 and 20). This complex was also induced in cells treated with TGFβ (data not shown) consistent with data that TGFβ represses Id-1 transactivation through this motif in HaCAT cells (27). Although these results suggest that this complex is ATF-3, commercially available antibodies did not result in disruption of this complex, and this complex was not competed with wild-type AT consensus oligonucleotide (data not shown). Therefore the polypeptides in this complex remain to be identified. Together these data suggest that HIF-1 only binds the Id-1 promoter in the absence of
ATF-3, and ATF-3 antagonizes HIF-1 binding to the Id-1 promoter.

**ATF-3 Regulates Differentiation of Hypoxic Neuroblastoma Cells**—Previous studies have demonstrated that long term, moderately hypoxic neuroblastoma cells (which would be predicted to have an activated UPR) down-regulate several ganglionic/neuronal genes (38). This has led to the proposal that hypoxia pushes neuroblastoma cells toward a more immature phenotype, resembling neural crest-derived neuronal precursor cells (13). Because down-regulation of Id family members is associated with neuroblastoma differentiation, we sought to determine whether previously described hypoxic responses were related to hypoxic up-regulation of Id-1 (and therefore the absence of the UPR in these cells).

As discussed previously, we found that the introduction of ATF-3 in hypoxic neuroblastoma cells blocks up-regulation of Id-1 (Fig. 3E). SK-N-BE(2)-plpc control cells (which induce Id-1 when hypoxic) and SK-N-BE(2)-ATF-3 expressing cells (which do not induce Id-1 when hypoxic) were rendered hypoxic for 16 h, and the expression of markers of differentiation was assessed by quantitative RT-PCR. As predicted from protein expression data (Fig. 3E), the presence of ATF-3 repressed Id-1 mRNA up-regulation in hypoxic SK-N-BE(2)-ATF-3 cells (Fig. 5). In contrast to hypoxic SK-N-BE(2)-plpc cells, the loss of the neuronal markers nfi and npy and induction of Sox9 were not seen in hypoxic SK-N-BE(2)-ATF-3 cells. Of note, the increase of nse in parental hypoxic cells was also blocked by the expression of ATF-3. While the nse promoter has conserved ATF and HIF-1 binding motifs, whether ATF-3 directly blocks HIF-1 transactivation of nse is unknown at this time. The down-regulation of gap43 seen in hypoxic SK-N-BE(2)-plpc cells was even more pro-
Announced in SK-N-BE(2)-ATF-3 cells; regulation of gap43 is primarily due to Nex1, a member of the NeuroD family (40). Inhibition of Nex1 by hypoxic-generated Id-1 would explain our observations, and this too deserves further study. Together, our data suggest that hypoxic regulation of Id-1 promote the loss of some neuronal markers in neuroblastoma, as well as the acquisition of at least one stem cell marker. However, the full effect of hypoxia on neuroblastoma differentiation is likely to differ with duration and severity of hypoxic incubation and is undoubtedly complicated by hypoxic activation of the other pathways including notch and AKT (41, 42).

DISCUSSION

We have established that Id-1 is highly regulated in hypoxic cells primarily at the transcriptional level. Our data demonstrate that in most cells Id-1 is down-regulated by hypoxia-induced ATF-3. Hypoxic repression of Id-1 (and perhaps other Id family members) would be predicted to promote cellular growth arrest. This may be a normal, physiological response by which hypoxic cells diminish energy needs. The Id proteins also play a crucial role in development. Bone marrow and lymph nodes are relatively hypoxic, and the role of HIF-1 in embryogenesis suggests physiological hypoxia occurs during embryogenesis (11, 43, 44). In the absence of hypoxia-induced ATF-3, Id-1 is up-regulated by HIF-1. One mechanism by which differentiation is blocked in some hypoxic cells (such as hematopoietic stem cells) and induced in others may be the hypoxic induction or repression, respectively, of Id family members.

In agreement with other recent findings we have found that in hypoxic cells PERK phosphorylates eIF2α leading to the induction of ATF-4 and ATF-4 targets including CHOP. eIF2α phosphorylation is also, in part, responsible for hypoxic up-regulation of ATF-3 and repression of Id-1. Remarkably, we have noted that the UPR is markedly diminished in hypoxic neuroblastoma cells. Functional ATF-4 and PERK are necessary for ras-transformed MEFs to grow as tumors in nude mice, and ATF-4 up-regulation has been noted histochemically in hypoxic human cervical cancer (32). However, the functional status of the UPR in other human cancers, many of which have mutations that allow them to tolerate cellular stresses, has not been well surveyed. In some cancers, such as neuroblastoma, the absence of a functional UPR may actually be beneficial to tumorigenesis. For example, the absence of the UPR in neuroblastoma cells also results in a failure of CHOP induction, a transcriptional target of ATF-4 and a reported pro-apoptotic protein (45).

The high basal phosphorylation of eIF2α we observed in neuroblastoma cells may be due to diminished expression of gadd34, an ATF-4 target that de-phosphorylates eIF2α and thus serves as a feedback loop (46). The significance of high basal eIF2α phosphorylation in neuroblastoma cells is unknown, but this observation may provide a clue to the aberrant hypoxic response in these cells. eIF2α phosphorylation normally results in the binding and inactivation of eIF2B, a GTP exchange factor necessary for eIF2 function and subsequent protein translation (47). Mutations in the regulatory subunits of the eIF2B complex, which have been generated experimentally and are also present in patients with various leukodystrophies, lead to decreased activity of eIF2B and render eIF2B resistant to inhibition by phosphorylated eIF2α, although many of these mutations appear to actually increase the stress response of these cells (48). It will be interesting to determine whether neuroblastoma cells have mutations in members of the eIF2B complex that render this complex immune to inhibition by phosphorylated eIF2α.

The identification of ATF-3 as a hypoxia-generated transcriptional repressor may provide insight into the mechanism of gene repression in hypoxic cells. Because Id-1 is up-regulated by HIF-1 and down-regulated by ATF-3, our findings demonstrate that ATF-3 can modulate HIF-1 activity. In addition, since ATF-3 is not induced in some hypoxic cells, our data demonstrate cell specific modulation of HIF-1 activity. While we noted up-regulation of Id-1 in hypoxic ATF-3−/− MEFs, the degree of induction (1.6-fold, Fig. 1D) was more modest than the induction of Id-1 in neuroblastoma cells (3.3-fold, Fig. 5) suggesting that other features may also contribute to the HIF-1 mediated up-regulation of Id-1 in hypoxic neuroblastoma cells. It is tempting to hypothesize that the antagonism of ATF-3 and HIF-1 may play a role in modulating expression of genes in addition to Id-1, and this may help explain some of the tissue and cell type differences in hypoxic response (Fig. 6).

HIF-1α is stabilized in hypoxic tumors and by oncogenic alterations often found in cancer and is an indicator of poor prognosis (reviewed in Ref. 1). HIF-1 transactivates glucose transporters, glycolytic enzymes, angiogenic factors, and other genes important for hypoxic adaptation to the tumor microenvironment (reviewed in Ref. 1). Whether Id proteins are HIF-1 targets in hypoxic tumors other than neuroblastoma is unknown. Id proteins play a vital role in proliferation, endothelial cell recruitment, and neo-vascularization in animal models, and Id-1 is a transcriptional repressor of thrombospondin, a negative regulator of angiogenesis (17, 18). Further studies are needed to determine both the phenotypic results of Id-1 down-regulation in hypoxic cells and whether the establishment of ATF-3, or the UPR, in hypoxic neuroblastoma cells can affect proliferation, angiogenesis, or differentiation in vivo through manipulation of Id-1.
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Acknowledgments—We thank H. P. Harding and D. Ron for their kind gift of many useful reagents including the PERK and ATF4 knock-out MEFs, T. Hai for ATF-3 knock-out MEFs, and R. Kaufman for the eIF2α S51A MEFs. We thank Francesca Ventura and Yibin Kang for supplying the Id-1 promoter/luciferase constructs. We also thank R. Alani, G. Kato, and M. Pagano and his laboratory, B. Dynlacht and his laboratory, and E. Skolnick for reagents. These experiments were initiated in the laboratory of Chi Dang, with his support and guidance and the assistance of Irina Chernysheva. We gratefully acknowledge Heather Harding, Jeffrey Z. S. Ye, and Gregory David for their many useful discussions. Heather Harding, Gregory David, Brian Dynlacht, Linda Lee, Simon Karpatkin, David Ron, and Ed Skolnick provided helpful criticisms in the preparation of this manuscript.

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