Hypoxia-inducible Factor-1 (HIF-1) Is a Transcriptional Activator of the TrkB Neurotrophin Receptor Gene*

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Neurotrophins and their cognate receptors play a pivotal role in the development and function of the nervous system. High expression levels of the neurotrophin receptor TrkB and its ligands in neuroblastomas are associated with an unfavorable outcome. We report here that NTRK2, which encodes the TrkB receptor tyrosine kinase, is an oxygen-regulated gene, whose expression is stimulated by the hypoxia-inducible factor-1 (HIF-1). TrkB mRNA and protein levels were elevated nearly 30-fold in neuroblastoma-derived Kelly cells in hypoxia (1% O₂) versus normoxia (21% O₂). A luciferase reporter construct containing ≈2.1 kilobases of the human TrkB promoter was activated about 6-fold both in hypoxia and after stimulation with the hypoxia mimetic 2,2′-dipyridyl (100 μM) at 21% O₂. Luciferase activity in the presence of 2,2′-dipyridyl was reduced significantly upon small interfering RNA knockdown of HIF-1α but not of HIF-2α. Accordingly, hypoxia failed to stimulate the TrkB promoter in mouse embryonic fibroblasts that lacked HIF-1α. The hypoxia-responsive promoter region could be mapped to three HIF-1 binding elements that were located between −923 and −879 bp relative to the transcription start site. The migration of cultured neuroblastoma cells was increased 2−fold upon incubation at 1% O₂. This effect of hypoxia was abrogated with the tyrosine kinase inhibitor K252a (200 nM). Our findings indicate that transcription of the NTRK2 gene is stimulated at low oxygen tension through a HIF-1-dependent mechanism. In conclusion, enhanced expression of TrkB could represent a critical switch for the previously reported dedifferentiation of neuroblastoma cells under hypoxic conditions.

Neurotrophins comprise a family of vertebrate-specific growth factors that have been studied extensively for their role in neuronal development (reviewed in Ref. 1). The cellular effects exerted by the neurotrophins, which include nerve growth factor, brain-derived neurotrophic factor (BDNF),3

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§The abbreviations used are: BDNF, brain-derived neurotrophic factor; HRE, HIF-1-binding elements; Chip, chromatin immunoprecipitation; DP, 2,2′-dipyridyl; NT, neurotrophin; HIF-1, hypoxia-inducible factor-1; MEF, mouse embryonic fibroblast; kbp, kilobase pair; EMSA, electrophoretic mobility shift assay; siRNA, small interfering RNA.
nisms that control its expression in neuroblastoma cells is desirable.

Local hypoxia due to an imbalance between oxygen supply to the neoplastic tissue and O₂ consumption by the tumor cells is a common situation in neuroblastomas and other malignancies (reviewed in Ref. 25). As a precondition for tumor progression, hypoxia triggers the formation of new blood vessels through the synthesis and release of vascular growth factors and their cognate receptors. Recent findings indicate that hypoxia can also modify gene expression in neuroblastoma cells toward a more immature and aggressive phenotype (26, 27). The molecular mechanisms that convey hypoxia-induced de-differentiation of neuroblastoma cells are poorly understood. Hypoxia-inducible factor-1 (HIF-1) exerts an important function in the genomic control at low oxygen tension. HIF-1 is a heterodimeric transcription factor consisting of an oxygen-regulated α-subunit and the constitutively expressed β-subunit (28). At normoxia the HIF-1α protein is post-translationally hydroxylated at two proline residues (29) and, following ubiquitination by the E3 ubiquitin ligase complex (30, 31), is rapidly degraded by the proteasome (32). Intriguingly, our sequence analysis of the promoter of the TrkB encoding gene, NTRK2, revealed several putative binding sites for HIF-1 in regulatory important regions. Furthermore, enhanced expression of TrkB in response to ischemic brain injury, a condition that is intimately linked with tissue hypoxia, has been reported (33). These evidences prompted us to hypothesize that TrkB expression may be controlled by oxygen tension. We demonstrate here that transcription of the TrkB encoding gene, NTRK2, is indeed stimulated in hypoxia through a mechanism that involves HIF-1. Enhanced expression of TrkB could play a role in the de-differentiation of neuroblastoma cells at low oxygen tension.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human neuroblastoma-derived Kelly cells (ACC 355), U2OS osteosarcoma cells (ATCC HTB-96), and human embryonic kidney (HEK) 293 cells (ACC 305), were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and the American Type Culture Collection, respectively. A HIF-1α-deficient mouse embryonic fibroblast cell line (MEF) was the kind gift of Roland H. Wenger (34). Kelly cells were maintained in RPMI 1640 medium, MEK293, U2OS, and MEF cells were grown in Dulbecco’s modified Eagle’s medium nutrient (PAA Laboratories, Pasching, Austria), each supplemented with 10% fetal calf serum (Biochrom KG, Berlin, Germany), 100 IU/ml penicillin, 1% glutamate, and 100 μg/ml streptomycin (all from Invitrogen). The cells were expanded until 40% confluency at 37 °C in a humidified atmosphere of 21% O₂, 5% CO₂. Hypoxic conditions were established by placing the cells in a tissue culture incubator (Binder GmbH, Tuttingen, Germany) at reduced oxygen content (1% O₂). In some experiments the iron chelator and HIF prolyl hydroxylase inhibitor, 2,2’-dipyridyl (DP, Sigma) was added at 21% O₂ to a final concentration of 100 μM.

**Reporter Gene Assays**—Kelly cells were grown to ~50% confluency in 6-well dishes, the MEF cells were transfected at 80% confluency in 24-well tissue culture plates. Nine hundred nanograms of the reporter constructs and 100 ng of a cytomegalovirus promoter-driven β-galactosidase plasmid were transiently co-transfected by the use of FuGENE 6® reagent (3 μl per well) according to the manufacturer’s protocol (Roche Diagnostics). Transfection with the empty pGL3basic vector (Promega) served as a control. The transfected cells were incubated for 16 h either at 1% or 21% O₂ prior to lysis in Reporter Lysis Buffer (Promega). Luciferase activities were measured in a luminometer (Microlite TLX1, MGM Instruments, Hamden, CT) and β-galactosidase activities were determined spectrophotometrically (Beckman DU 540 spectrophotometer, Beckman Coulter GmbH, Krefeld, Germany) as described (35, 36). Data are presented as relative light units normalized to β-galactosidase activities for the control of transfection efficiencies.

**siRNAs and Transfection Procedures**—The use of siRNAs for the knockdown of HIF-α transcription factors in cultured cells has been described in detail elsewhere (37). HIF-α siRNAs with the following sequences were synthesized by Xeragon/Qiagen (Köln, Germany): HIF-1α, 5’-GCCACUUGAAGUGCCU-AdTdT-T’3’ (sense) targeting 1378 to 1398 bp of the human HIF-1α mRNA (NCBI accession number AB304431.1); and HIF-2α, 5’-GCGACACGUGAGUAAGAAdTdT-T’3’ (sense), targeting 2274 to 2294 bp of the human HIF-2α mRNA (NCBI accession number NM_001430.1). As a negative control, we used siRNA targeting the firefly luciferase coding sequence of the pGL2basic vector (38): 5’-CGUACGCGAUAUCUG CG-AdTdT-T’3’ (sense); synthesis by Eurogentec, Brussels, Belgium. Kelly cells were co-transfected at 40–50% confluency in 24-well plates with the TrkB promoter-reporter construct pGL3-TrkB-2107 (see below) and a cytomegalovirus promoter-driven β-galactosidase plasmid as described above. On the next day, the cells were transfected with the siRNAs (200 nM final concentration) using Oligofectamine (Invitrogen) according to the supplier’s protocol. Twenty-four hours after siRNA transfection, the cells were stimulated with the hypoxia-mimetic DP (100 μM) for 16 h.

**Reverse Transcription Real Time PCR**—Total RNA was isolated from cultured cells by the use of TRIzol® reagent (Invitrogen) according to the manufacturer’s protocol. First-strand cDNA synthesis was performed with 2 μg of total RNA using oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen). Two percent of the volumes of the reaction products were used for quantitative real time PCR amplification with SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA). The PCRs were carried out on a GeneAmp5700 thermocycler (PerkinElmer Life Sciences) under the following conditions (45 cycles): DNA denaturation (15 s) at 94 °C, primer annealing (15 s) and extension of double-stranded DNA at 60 °C (60 s), detection of SYBR Green fluorescence at 77 °C (30 s). The PCR primers used for the amplification reactions are listed in Table 1. The C_t values for the genes of interest were subtracted from the C_t values for β-actin to obtain ΔC_t values. Differences in transcript levels between normoxic (21% O₂) and hypoxic (1% O₂) or DP-treated cells were calculated as ΔΔC_t (normoxia) = ΔC_t (hypoxia/DP). -Fold increases in mRNA levels were obtained according to 2^-ΔΔC_t.

**Plasmids**—Cloning of the upstream region of the human gene, NTRK2, which encodes the TrkB neurotrophin receptor...
(NCBI accession number AL390777) from a bacterial artificial chromosome (clone RP11–301F14) has been described previously (16). The plasmid pGL3-TrkB-2107 contained a ～2.1 kilobase pair (kb) ScaI restriction fragment of NTRK2 extending from -2107 to +104 bp relative to the transcription start point in the ScaI site of the pGL3basic reporter vector (Promega). This plasmid was identical to the previously described pTrkBprom construct (16) except that it contained the pGL3basic vector backbone instead of the pGL2basic vector. A 5'–truncated TrkB promoter construct (−969 bp to +104 bp) was obtained by restriction digest of pGL3-TrkB-2107 with KpnI and designated pGL3-TrkB-969. Another truncation at the 5'–end was generated by PCR amplification by the use of two primers (pQbL, Erlangen, Germany) and the following oligonucleotides: 5'–CATCGTACCTGGAATCCCGAC-ATGCTGTGCTG-3' (forward), 5'–GTCTCCTTCGCACTGATCTG-3' (reverse) (double mutant forward), 5'–GTTCACATGCCTGACACACT-3' (reverse). The forward primer contained a suffixed KpnI restriction site allowing ligation of the amplicon with the 5'–end of the pGL3basic plasmid promoter from -969 bp to -104 bp. A PCR-based protocol was applied to introduce site-directed mutations into three identified HIF-1-binding elements (HREs) that were located between -923 bp and -879 bp in the promoter of the TrkB gene. For this purpose the following PCR primers (mutant nucleotides underlined) were used with pGL3-TrkB-969 serving as DNA template: 5'–TGAGGCTCGGGAGCAGGCTGGC-AGCCGGAGA-3' (forward), 5'–GGCTCCATCTTCCAGCGGATA-3' (reverse), 5'–GATGTGTGCTATATGTGC CGCGCTTATGTGCGCGCTT-3' (double mutant forward), 5'–GGCTCCATCTTCCAGCGGATA-3' (reverse). The resulting mutant plasmid pGL3-TrkB-ΔHRE2/3 served as template for a third PCR mutation: 5'–TGAGGCTCGGGAGCAGGCTGGC-AGCCGGAGA-3' (forward), 5'–GGCTCCATCTTCCAGCGGATA-3' (reverse), 5'–CGACCGGCTC GTCTATGCTAC-3' (mutant forward), 5'–GGAGCAGTAGATTAGCCTCGG-3' (mutant reverse). The amplified DNA was ligated into the KpnI–SacI sites of the pGL3basic plasmid yielding pGL3-TrkB-ΔHRE1/2/3. The correct identity of the amplified DNA was assessed by automated sequencing (MWG Biotech, Ebersberg, Germany).

**TABLE 1**

| Gene       | NCBI accession No. | Sequence |
|------------|-------------------|----------|
| Human TrkB| Q16620             | Forward: 5'–CTCTACTCTACACTTGGAAGAA-3' |
| Human TrkA| BC06250            | Reverse: 5'–ATGCTGTCCTCACCAGGTCACG-3' |
| Human TrkB| BC013693          | Forward: 5'–GGTTCCATCTTCCAGCGGATA-3' |
| Human p75NTR| BC052977       | Reverse: 5'–QQCCATCTTCGAGCTCGAAATCC-3' |
| Human β-actin| NM_001101     |          |

**Electrophoretic Mobility Shift Assay (EMSA)**—Electrophoretic mobility shift assays were carried out with nuclear extracts from Kelly cells, which had been grown on 10-cm dishes for 5 h at 1 or 21% O2, respectively. Nuclear extract preparation and DNA binding reaction were performed according to Ref. 41. In brief, the oligonucleotides (see below) were 5'–end labeled with [γ-32P]ATP (7,000 Ci/mmol, catalog number 35020, ICN Biochemicals, Eschwege, Germany) and incubated at room temperature for 30 min with 5 μg of nuclear extract in 1× reaction buffer consisting of: 12 mm Hepes, pH 7.9, 4 mm Tris-HCl, pH 7.9, 60 mm KCl, 1 mm EDTA, 1 mm phenylmethylsulfonyl fluoride, 1 μg of denatured calf thymus DNA (Sigma). The following double-stranded oligonucleotides with the putative HIF-1 binding elements in the TrkB pro-
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moter were used: 5′-GACACACACACGCACGGGTGTCGATGTCTA-3′ (TrkB-HRE1), 5′-GACACACAAAAAACACGGCGAAACATGTCTA-3′ (TrkB-HRE1mut), 5′-GAGTGTGTCGTTGCGCAGGTGTGTAAGA-3′ (TrkB-HRE2/3), 5′-GATGTGTCGAAATGTGCAGAAAATGTGAA-3′ (TrkB-HRE2/3mut). For the supershift experiments 1 μg of a mouse monoclonal anti-HIF-1α antibody (catalog number 610958, BD Biosciences) was added to the binding reactions overnight at 4 °C. The samples were run for 4 h on a non-denaturing 4% polyacrylamide gel at 4 °C. The dried gels (gel dryer, model 583, Bio-Rad) were exposed to an x-ray film for 1 h.

Chromatin Immunoprecipitation Assay (ChIP)—ChIP assays were carried out with a commercial kit (Upstate Biotechnology, Hamburg, Germany) according to our previously described protocol (36, 42). In brief, Kelly cells were incubated for 5 h at 1 or 21% O₂, respectively. Cross-linking of DNA-bound protein was achieved by 10 min incubation of the cells in a 1% formaldehyde solution at room temperature. After quenching with 125 mM glycine the cell lysates were ultrasonicated yielding an average DNA fragment size of 200 to 600 bp. Immunoprecipitations were performed overnight at 4 °C using the following antibodies: mouse monoclonal anti-HIF-1α antibody (catalog number 610958, BD Biosciences), rabbit polyclonal anti-HIF-1α antibody (catalog number 100–449, Novus Biologicals), and rabbit polyclonal anti-acetylated histone H3 antibody (catalog number 06-599, Upstate Biotechnology). Incubation with normal mouse IgG served as a negative control. The antibody-bound proteins were precipitated for 3 h at 4 °C with DNA-blocked protein A/G-agarose (Upstate Biotechnology). After several washes in low and high salt buffer, the DNA was eluted from the agarose beads, extracted by phenol:chloroform treatment, and precipitated in 100% ethanol. The DNA pellet was resuspended in 30 μl of TE buffer, and 1/10 volume was taken for PCR amplification of the human TrkB promoter by the use of the following primers: 5′-GAGGACGACGACGCAGCACG-AGGATAG-3′ (forward), 5′-GCAGATCTGTTGAAAAGGACGACGCTGTTGAG-3′ (reverse). The PCR (32 cycles) was carried out on a thermocycler (GeneAmp2400, Applied Biosystems) with DNA denaturation at 95 °C, primer annealing at 58 °C, and primer extension at 72 °C, each step lasting 30 s. The amplicons were separated on a 1.2% agarose gel and visualized with SYBR Safe® DNA stain (Invitrogen).

Cell Migration Assay—Assays were performed on polycarbonate filters with 8-μm pore size following the manufacturer’s instructions (Biorad® Cell Culture Inserts, BD Biosciences). Briefly, Kelly cells that had been incubated for 24 h either at 21 (controls) or 1% O₂ to stimulate TrkB expression were detached from the tissue culture dishes with Accutase (catalog number L11–007, PAA). The cells were seeded in serum-free medium at 77,000 cells per well in the upper compartment of the transwell chambers. Tissue culture medium with or without supplementation of 50 ng/ml human BDNF (catalog number B-250, Alomone Labs, Jerusalem, Israel) was added to the lower compartments. In some experiments, the tyrosine kinase inhibitor K252a (catalog number 420298, Calbiochem, Darmstadt, Germany) was given to the cells at a final concentration of 200 nm. Cell migration was assessed in a 21% O₂, 5% CO₂ atmosphere at 37 °C. After 27 h of incubation, the migrated cells were stained with toluidine blue (1% solution in 1% sodium tetaborate, Morphisto GmbH, Frankfurt, Germany) on the lower side of the membranes. The cells were counted under the microscope (Axiovert S100, Carl Zeiss AG, Göttingen, Germany) at ×100 magnification. The migration assays were carried out in triplicate and at least three independent experiments were performed for each condition.

Statistics—If not otherwise indicated all values are presented as mean ± S.D. Student’s paired t test was applied to reveal statistical significances. p values less than 0.05 were considered significant.

RESULTS

The Kelly neuroblastoma cell line was used to explore whether expression of the different neurotrophin receptors is sensitive to changes in local oxygen tension. Kelly cells were chosen because they have previously been used as a model to study the molecular mechanisms of oxygen-regulated gene expression (43). Furthermore, high expression levels of the TrkB neurotrophin receptor and its cognate ligands, BDNF and NT-4/5, in neuroblastomas are associated with aggressive tumor growth (18, 19). Compared with normoxia (21% O₂) TrkB transcript levels were increased almost 30-fold in Kelly cells that had been incubated under hypoxic conditions (1% O₂) for 16 h (Fig. 1A). Likewise, treatment with the hypoxia mimetic DP (100 μM) for 16 h at 21% O₂ stimulated TrkB expression ~10-fold (Fig. 1A). In contrast, expression of the TrkA and TrkC receptor tyrosine kinases and the low affinity neurotrophin receptor p75NTR was not significantly changed by the applied protocols (p > 0.05, n = 4). The U2OS osteosarcoma line and HEK293 cells were used to explore whether stimulation of TrkB expression at hypoxia was confined to neuronal tumor cells (Kelly) or whether it represents a regulatory mechanism that is preserved among cells of different tissue origin. As shown in Fig. 1B, TrkB transcript levels were increased significantly in U2OS and HEK293 cells in hypoxia (1% O₂, 16 h). Accordingly, treatment with DP (100 μM) for 16 h stimulated TrkB expression at 21% O₂ in all three cell lines (Fig. 1B). Compared with Kelly cells, the increase of TrkB mRNA in response to hypoxia and DP was weaker in HEK293 and U2OS cells (Fig. 1B). To analyze the expression kinetics of TrkB in hypoxic Kelly cells, we took samples at 4, 8, 16, 24, and 32 h, respectively, after lowering the ambient oxygen to 1% O₂. Representative results of these experiments, which were repeated as triplicates twice, are shown in Fig. 2A. TrkB mRNA levels increased continuously between 4 and 24 h of exposure to 1% O₂ before reaching a plateau during longer incubation periods. To exclude potential adverse effects of extended hypoxia, i.e., changes of the pH in the culture medium, the cells were incubated at 1% O₂ for 16 h in all subsequent experiments. We performed immunoblot analysis to investigate whether the rise of TrkB mRNA in hypoxic Kelly cells was accompanied by a similar increase in TrkB protein. Using a monoclonal anti-TrkB antibody, the two 145- and 95-kDa isoforms, gp145TrkB and gp95TrkB, could be detected in membrane preparations of Kelly cells grown for 16 h at 1% O₂ (Fig. 2B). Hypoxic stimulation of the cells is indicated by their robust expression of the HIF-1α protein, which was not detectable in Kelly cells at 21% O₂ (Fig. 2B). Likewise, no TrkB protein
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Kelly cells and the HIF-α isoforms were silenced by transfection of specific HIF-1α and HIF-2α siRNAs (37). The transfected cells were stimulated with DP (100 μM) for 16 h. TrkB promoter-driven luciferase activities were increased more than 6-fold in DP-treated versus untreated Kelly cells that had been transfected for control with the luc-siRNA (Fig. 3A). TrkB promoter activation in the presence of DP was reduced significantly (p < 0.05, n = 6) by transfection of HIF-1α siRNA, whereas silencing of HIF-2α had no significant effect (Fig. 3A). The efficiency of siRNA knock-down was assessed by immunoblotting with antibodies against HIF-1α and HIF-2α. As expected, the HIF-1α and HIF-2α proteins were markedly reduced in the respective siRNA-transfected cells (Fig. 3B). However, both proteins could still be detected in the siRNA-treated cultures indicating that gene silencing was incomplete (Fig. 3B). Similar results were obtained with another set of HIF-1α and HIF-2α siRNAs (45, 46) (data not shown). These findings suggest that TrkB expression in hypoxic Kelly cells is stimulated by HIF-1 rather than by HIF-2.
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To further study the role of HIF-1 in TrkB expression at low oxygen tension we took advantage of a MEF cell line that lacks HIF-1α (34). HIF-1α-deficient MEF cells and the corresponding wild-type cell line, which carries both HIF-1α alleles, were transiently transfected with the ~2.1-kbp TrkB promoter reporter construct pgL3-TrkB-2107 and subsequently maintained for 16 h either at 1 or 21% O₂. TrkB promoter activity was enhanced more than 3-fold upon exposure of the transfected wild-type cells (MEF HIF-1α⁺/⁺) to hypoxia (p < 0.05, n = 5) (Fig. 4). In contrast, incubation at 1% O₂ did not significantly (p > 0.05) change TrkB promoter activity in HIF-1α-depleted MEF cells (MEF HIF-1α⁻/⁻). Control experiments were performed by transfection of the pgL3basic vector, which was not inducible in hypoxia as expected (Fig. 4). These results show that HIF-1α is necessary for the stimulation of the TrkB promoter at reduced oxygen tension. It is noteworthy that MEF cells contained very low levels of endogenous TrkB transcripts, which did not increase at 1% O₂ in both, wild-type and HIF-1α⁻/⁻ cells (data not shown).

Next we aimed at identifying the cis-element(s) required for activation of the TrkB promoter at low oxygen tension. A computer-based analysis (Transfac®) of the TrkB promoter sequence (NCBI accession number AL390777) revealed 12 putative binding sites for HIF transcription factors with the core sequence 5′-RCGTG-3′ (47). These elements were located between −1967 and −35 bp upstream of the transcription start site in the NTRK2 gene. To pinpoint the functional transcription factor binding sites, the ~2.1-kbp TrkB promoter reporter construct was used as a template for generating several shorter plasmids with truncated 5′-ends. These constructs were transiently transfected into Kelly cells, which were then exposed either to 1 or 21% O₂. Exposure to low ambient oxygen (1% O₂) activated the pgL3-TrkB-969 reporter construct almost 5-fold (Fig. 5A), whereas pgL3-TrkB-878 was not significantly stimulated in hypoxia (p > 0.05, n = 3). Activity of the pgL3basic vector, which served as a negative control, was not enhanced at 1% O₂ (data not shown). With these transfection experiments, the hypoxia-sensitive region in the TrkB promoter could be allocated to a 91-nucleotide sequence extending between −969 and −878 bp relative to the transcription start site. This sequence contained three putative HIF binding elements designated HRE1 through HRE3 (Fig. 5B). ChIP analysis was performed to explore whether HIF-1 interacts with the TrkB promoter in a natural chromosomal configuration. In vivo binding of HIF-1α to the TrkB promoter was detected in Kelly cells that had been incubated at 1% O₂ for 5 h (Fig. 5C). As expected, no HIF-1α interaction with the TrkB promoter occurred in Kelly cells at 21% O₂ (Fig. 5C). EMSAs were performed with nuclear extracts from hypoxic (1% O₂, 5 h) Kelly cells to test whether HIF-1 binds to the identified HREs in the TrkB promoter. Two different oligonucleotides were initially used for the EMSA experiments: oligonucleotide TrkB-HRE1 contained the predicted
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We examined whether inactivation of the identified HIF-1-binding elements would impair the transcriptional response of the pGL3-TrkB-969 construct to hypoxia. For this purpose, the same base pairs were mutated in the TrkB promoter reporter construct as in the EMSA probes (Fig. 6B). Compared with the wild-type construct, pGL3-TrkB-969, the combined mutation of HRE2 and HRE3 resulted in a more than 50% reduction of reporter activity in hypoxia (1% O2). This decrease became statistically significant upon additional disruption of HRE1 in the pGL3-TrkB-969 plasmid (Fig. 7). The mutant construct, which lacked HRE1 through HRE3, could also not be activated by treatment of the transfected Kelly cells with 100 μM DP (data not shown).

Finally, we examined the functional consequences of TrkB expression in neuroblastoma-derived Kelly cells in hypoxia. Previous studies demonstrated that the BDNF-TrkB signaling pathway regulates cell migration in the nervous system and non-neuronal tissues (49–51). We therefore examined whether exposure to hypoxia would affect the migratory potential of Kelly cells. For this purpose, migration assays were performed on cells that had been grown for 24 h either at 1 or 21% O2 to stimulate TrkB expression. As shown in Fig. 8, the number of migrating cells was increased −2-fold (p < 0.05, n = 5) in Kelly cells that had been pre-exposed to 1% O2 compared with 21% O2. Cell migration in hypoxia and at 21% O2 was reduced significantly in the absence of the TrkB ligand, BDNF (Fig. 8). BDNF was usually added as a chemoattractant to the culture medium of the lower transwell compartment. Treatment with the tyrosine kinase inhibitor K252a (200 nM) also reduced the number of migrating cells significantly both in normoxia and hypoxia (Fig. 8). These results indicate that up-regulation of TrkB expression is responsible, at least in part, for the enhanced migratory potential of neuroblastoma cells in hypoxia.

DISCUSSION

In this report, we show that transcription of the NTRK2 gene, which encodes the TrkB neurotrophin receptor, is activated at low oxygen tension through a mechanism that involves HIF.
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FIGURE 6. A, EMSA demonstrating binding activity in nuclear extracts of hypoxic (1% O₂, 5 h) Kelly cells to the predicted HRES in the human TrkB promoter. The binding reactions were carried out at room temperature for 30 min with 5' end labeled oligonucleotides and 5 μg of nuclear extract. The samples were loaded on a non-denaturing 4% polyacrylamide gel, which was run for 4 h at 4 °C. Complex formation was visualized by autoradiography of the dried gels. In addition to the appearance of nonspecific (ns) and constitutive signals (c), a retarded band (arrow, shift) was obtained with nuclear extract of hypoxic (1% O₂) Kelly cells (lanes 3 and 11). This retardation band was not detected with normoxic (21% O₂) cell nuclear extracts (lanes 2 and 10). The complexes could be supershifted (arrow, supershift) by incubation with specific anti-HIF-1α antibody (lanes 4 and 12). Disruption of the HIF recognition sequence in two mutant oligonucleotides (TrkB-HRE1mut and TrkB-HRE2/3mut) resulted in a loss of specific binding activity in the hypoxic Kelly cell nuclear extracts (lanes 7 and 15). B, sequence of the oligonucleotides that were used for the EMSA experiments. The HRE core sequences and the corresponding mutations are underlined and indicated in bold.

HIFs have been recognized as a family of transcription factors, which act as master regulators of gene expression in hypoxia. We took advantage of the siRNA technology to address the question of whether transcription of the NTRK2 gene is presumably stimulated by HIF-1 or HIF-2. This distinction is relevant as HIF-1 and HIF-2 differ in their cellular tissue distribution and target gene preference suggesting specific roles for both proteins in physiology and disease (52–55). Even though complete knock-down of the two molecules could not be accomplished, our results strongly suggest that transcription of the NTRK2 gene in response to hypoxia is mediated through HIF-1 rather than HIF-2. This conclusion is supported by the finding that activation of the TrkB promoter at low oxygen tension in embryonic fibroblasts required HIF-1α.

HIFs commonly interact with HREs with the core sequence 5'-RCGTG-3' (see Ref. 47, reviewed in Ref. 56). However, a single HRE is frequently not sufficient to convey hypoxia sensitivity to a promoter unless adjacent transcription factor binding sites are present (56). Remarkably, the 2-kbp upstream region of the NTRK2 gene contains as many as 12 consensus HREs, some of which are clustered in regulatory active domains. We demonstrated in a recent study that the Wilms tumor protein Wt1 stimulates transcription of the TrkB promoter through binding to a GC-rich consensus motif, which is located in proximity to the herein identified HREs (16). It is therefore conceivable that HIF-1 and Wt1 act synergistically in regulating TrkB expression.
Up-regulation of TrkB in hypoxia could have major implications for the pathology of neuroblastoma and the biology of non-malignant cells. Neuroblastomas, which are composed mainly of immature sympathetic neural precursors, often with remaining neural crest traits (17, 57), arise during development of the sympathetic nervous system. The expression of neurotrophin Trk receptors in neuroblastomas has been characterized as a reliable prognostic factor for the heterogeneous clinical behavior of the tumors. Thus, TrkB and its major ligand BDNF are predominantly expressed in highly malignant neuroblastomas, whereas TrkA is present in more favorable neoplasms with better outcome (18, 58). Strikingly, neuroblastoma cells respond to ambient hypoxia with a more immature gene expression program and transformation into stem cell-like phenotypes (59). Our current findings suggest that up-regulation of TrkB in neuroblastoma cells is part of the genomic response to local tissue hypoxia, which may trigger an aggressive tumor biology.

Several mechanisms can be envisaged, by which the TrkB neurotrophin receptor promotes a malignant behavior of neuroblastomas. Thus, TrkB signaling was found to promote tumor cell survival and prevent neuroblastoma cells from chemotheraphy-induced apoptosis through activation of the phosphatidylinositol 3-kinase/Akt pathway (21–23). Anoikis is a type of apoptosis, which is initiated when tumor cells become detached from their extracellular matrix support. As such, anoikis may function as a physiological barrier to prevent metastasis. In an unbiased genome-wide screen to identify metastasis-associated genes the TrkB neurotrophin receptor was found to suppress caspase-dependent anoikis of non-malignant epithelial cells via the phosphatidylinositol 3-kinase/protein kinase B signaling cascade (60). It is therefore plausible, although not proven yet, that activation of specific pro-survival mechanism(s) contributes to the high metastatic potential of TrkB expressing neuroblastomas.

Our own findings demonstrate that up-regulation of TrkB in hypoxia increases the migratory potential of neuroblastoma cells. Consistently, cell migration was virtually blunted during treatment with the tyrosine kinase inhibitor K252a and upon removal of the TrkB ligand BDNF from the lower compartment of the transwell chambers. Cell migration is a critical step during normal tissue formation and a precondition for tumor invasion. Therefore, it is conceivable that TrkB expression in neuroblastoma reflects an immature state, which enables the tumor cells to acquire a migratory and thus invasive phenotype.

Angiogenesis, the formation of new blood vessels from pre-existing ones, is another important event in tumor progression. Vascularization of neuroblastomas and their content of pro-angiogenic molecules, i.e. vascular endothelial growth factor, correlate with a poor clinical outcome (61, 62). We reported previously that TrkB is necessary for blood vessel development in embryonic mouse hearts (16). In a recent study, activation of TrkB through BDNF was found to increase HIF-1α in neuroblastoma cells thereby stimulating vascular endothelial growth factor expression (63). These combined data suggest that hypoxia, through HIF-1α-dependent up-regulation of TrkB, may induce a pro-angiogenic switch in neuroblastoma. Our findings could also be relevant to other cancer types because, obviously, hypoxic stimulation of TrkB was not restricted to neuroblastoma-derived Kelly cells, but likewise occurred in an osteosarcoma line and in transformed human embryonic kidney cells.

Preliminary data indicate that low ambient oxygen stimulates TrkB expression also in adult rats in vivo suggesting that hypoxia may be a physiological signal for TrkB in normal tissues as well (4). Concordantly, local expression of the TrkB neurotrophin receptor was enhanced after ischemic/hypoxic brain injury in young rats (33). It remains to be clarified whether up-regulation of TrkB in vivo has a protective effect against hypoxia in neuronal and other tissues.

Finally, our results provide novel insights into the transcriptional regulation of the TrkB encoding gene, NTRK2. The overall significance of the BDNF-TrkB pathway for the development and function of the nervous system requires that the spatio-temporal expression of the receptor and its ligand are precisely fine-tuned. With the identification of hypoxia-responsive elements that mediate susceptibility to variable oxygen tension we discovered a novel regulatory switch for TrkB expression, in addition to the previously identified transcriptional regulators (59–61).

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