Hypoxia-inducible Factor-1α Enhances Haptoglobin Gene Expression by Improving Binding of STAT3 to the Promoter*

Mi-Kyung Oh†, Hyo-Jung Park‡, Nam-Hoon Kim†, Seon-Joo Park‡, In-Yang Park‡, and In-Sook Kim†,†

From the Departments of †Medical Lifescience and ‡Obstetrics and Gynecology, College of Medicine, The Catholic University of Korea, Seoul 137-701, Korea

Haptoglobin (Hp) is known to play a role in angiogenesis as well as in anti-inflammation. STAT3 is a major transcription factor for expression of human Hp. We investigated whether hypoxia-inducible factor-1α (HIF-1α), a key mediator of angiogenesis, participates in Hp gene expression. HIF-1α overexpression by gene transfection or hypoxia augmented Hp transcription in HepG2 human hepatoma cells. Conversely, knockdown of HIF-1α by specific siRNA transfection diminished Hp expression, although the level of STAT3 phosphorylation remained unchanged. A luciferase reporter assay using mutant Hp promoters demonstrated that two adjacent DNA elements, a STAT3-binding element (SBE) and a cAMP-response element (CRE)-like site in human Hp promoter −120/−97, were required for HIF-1α-stimulated transactivation of the Hp gene. HIF-1α, STAT3, and p300/CBP were simultaneously bound to the SBE/CRE as a complex form. When HIF-1α was knocked down, STAT3 binding to the SBE in the Hp promoter was attenuated. Our findings suggest that HIF-1α assists STAT3 in strong binding to the proximal SBE in the Hp promoter. The CRE-like site located near the SBE may contribute to the formation of a stable complex of STAT3, HIF-1α, and p300/CBP, which leads to maximum transcription of the Hp gene.

During the inflammatory process, acute phase reactions progress sequentially to prevent ongoing damage and to activate tissue repair (1). Haptoglobin (Hp) is a major acute phase protein, and its concentration in the circulating blood increases 2–4-fold in several pathologic conditions, such as trauma, cancer, and pregnancy (2). Hp binds strongly to extravascular Hb and rapidly removes the toxic Hb via the CD163 scavenger receptor of macrophages (3). Consequently, Hp protects tissues from Hb-stimulated oxidative damage (4, 5). Hp has also been reported to function as an angiogenic factor and a cell migration factor (6–8). Recently, we found that Hp improves the angiogenic property of endothelial progenitor cells and promotes the recovery of blood perfusion after ischemic injury (9). These findings suggest that Hp may participate in the vascular remodeling for tissue repair after vascular damage.

The expression of Hp is tissue-specific, occurring primarily in the adult liver. Human hepatic Hp expression is induced principally by IL-6-type cytokines that activate STAT3 (signal transducer and activator of transcription 3), a major transcription factor of Hp (10, 11). STAT3 has also been reported to induce expression of hypoxia-inducible factor-1α (HIF-1α), a key transcription factor induced by hypoxia (12, 13). Under hypoxic conditions, STAT3 and HIF-1α bind simultaneously to the VEGF promoter via formation of a transcription complex, which consists of STAT3, HIF-1α, the transcription coactivator p300/CBP, and Ref-1 (redox effector factor-1) (14, 15). These findings suggest that HIF-1α associates with STAT3 to lead to maximum transcription of specific genes. This raises the possibility that HIF-1α may act cooperatively with STAT3 for transcription activation of the Hp gene. The role of HIF-1α in Hp gene expression was previously unknown.

There are two consensus STAT3-binding elements (SBEs) in the 5′-flanking region of the Hp gene, a proximal SBE at positions −120 to −112 and an upstream SBE at positions −254 to −246. However, only the proximal SBE is required for Hp gene expression (10, 16). This suggests that structural components around the two SBEs may affect the binding of STAT3 and the formation of an active transcription complex. IL-6-response elements (IL-6REs) in the 5′-flanking regions of several genes consist of two adjacent cis-acting regulatory elements, a SBE and a cAMP-response element (CRE), and STAT3 binds in combination with CRE-binding proteins for target gene expression (17, 18). In the Hp promoter, a CRE-like site is located 7 bp from the proximal SBE, and this SBE/CRE corresponds to the IL-6RE. However, the role of the CRE-like site near the proximal SBE in the transcription of the Hp gene has not been established.

To verify the effect of HIF-1α on hepatic Hp gene expression, we investigated whether HIF-1α enhances Hp gene expression by acting cooperatively with STAT3 on the proximal SBE/CRE found in the Hp promoter.

**EXPERIMENTAL PROCEDURES**

Cell Culture—HepG2 cells were cultured in RPMI 1640 medium (JBI WelGENE, Daegu, Korea) supplemented with 10% fetal bovine serum (Gibco Laboratories, Gaithersburg,
Cooperation of HIF-1α and STAT3 in Haptoglobin Expression

MD). In experiments with IL-6 stimulation, the cells were treated with 5 or 10 ng/ml human recombinant IL-6 (rIL-6; R&D Systems, Minneapolis, MN). To inhibit STAT3 phosphorylation, the cells were pretreated for 30 min with 1 μM auranofin (Alexis, Lausen, Switzerland) before stimulation with 10 ng/ml rIL-6. For hypoxic stimulation, HepG2 cells were incubated for 5 h in a hypoxic chamber equilibrated with a gas mixture containing 1% O₂ and 5% CO₂ (Thermo Forma, Marietta, OH).

**Transient Transfection of Cells**—To prepare HIF-1α-overexpressing cells, HIF-1α cDNA subcloned into a pEGFP vector (Professor Kyu-Won Kim, Seoul National University, Seoul, Korea) was transfected into HepG2 cells using Lipofectamine 2000 transfection reagent (Invitrogen), according to the manufacturer’s protocol.

**Western Blot Analysis**—Cells were lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM phenylmethylsulfonil fluoride) for 30 min on ice. Equal amounts of protein were separated on a 12% SDS-polyacrylamide gel and visualized with the following human antibodies: anti-Hp (Sigma), anti-fibrinogen (Sigma), anti-HIF-1α (Novus Biologicals, Littleton, CO), anti-phospho-STAT3 (Cell Signaling Technology, Boston, MA), anti-STAT3 (Cell Signaling), and anti-CCAAT/enhancer-binding protein-β (C/EBPβ; Santa Cruz Biotechnology, Heidelberg, Germany). The proteins to be analyzed were visualized using an enhanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK).

**RT-PCR**—Total RNA was isolated using RNA STAT-60™ (Tel-Test Inc., Gainesville, FL). 2 μg of total RNA was reverse-transcribed into cDNA, followed by PCR amplification using the following specific primers: Hp, 5′-GTGGGACTCGAGGCAATGATC-3′ (forward) and 5′-GCATTAGTTCTCAGCTACTGTTCT-3′ (reverse); fibrinogen 5′-ACACATACTCGACCACTAAGTATTTGATA-3′ (forward) and 5′-CTCCCCCATTTGGAATGTCTGTTTCT-3′ (reverse); GAPDH, 5′-ACACAACTGCTCAGCAGGACTACAGGATGCTTTC-3′ (forward) and 5′-CCGACCTAAGTAAAGCCATCAGGTCTCT-3′ (reverse). Products were separated by electrophoresis on a 1% agarose gel and visualized by staining with ethidium bromide.

**HIF-1α RNA Interference**—HIF-1α siRNA was purchased from Dharmacon (Lafayette, CO) and introduced into HepG2 cells according to the manufacturer’s protocol. Cells were plated at a density of 4 × 10⁵ cells/well in a 6-well plate and cultured for 24 h. The siRNA for HIF-1α (200 μM) was mixed with 4 μl of DharmaFECT™ transfection reagent and incubated for 15 min at room temperature. The transfection mixture was added to the cell culture and incubated for 24 h.

**Preparation of Plasmid Constructs**—Genomic DNA was extracted from human umbilical vein endothelial cells using the LaboPass™ genomic DNA extraction kit (Cosmo Genetech, Seoul). Hp promoter region −994/+26 was amplified by PCR and ligated into the pGEM-T-Easy vector (Promega, Madison, WI). The promoter region was re-amplified using the following primers with KpnI and XhoI restriction sites: 5′-ACTGGTGACTGAAAGGTTACCTTCTCGGAACTTGGAATTGTA-3′ (forward) and 5′-ACTCTGCCAGGTTGTCCTGCCTGGAAGAGCA-3′ (reverse). The PCR-amplified 1020-bp Hp promoter fragment was subcloned into the pGL3-luciferase reporter vector at the KpnI/XhoI sites (Promega). Serial 5′-deleted Hp promoters were prepared by PCR amplification using the same reverse primer containing the XhoI restriction site and the following forward primers containing KpnI restriction site and the following forward primers containing KpnI restriction site: Hp promoter −529/+26, 5′-ACTGGTACCTTGGAAAAGACAT-3′ (forward) and 5′-ACTGGTACCTTGGAAAAGACAT-3′ (reverse). The luciferase reporter vectors were subcloned into the pGL3-luciferase reporter vector at the KpnI/XhoI sites.

**Site-directed Mutagenesis**—The regions of the SBE and CRE-like site in Hp promoter promoter −183/+26 were specifically mutated using an oligonucleotide QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The specific mutations were confirmed by DNA sequencing (Macrogen Inc., Seoul).

**Luciferase Assay**—HepG2 cells were plated in 12-well plates at a density of 3.0 × 10⁵ cells/well and incubated for 24 h. The cells were cotransfected with the reporter constructs along with the HIF-1α cDNA-containing plasmid. After 24 h, the cells were lysed, and luciferase activity in cell extracts was determined using a luciferase assay kit (Promega). The transfection efficiency was normalized to cotransfected β-galactosidase activity.

**Electrophoretic Mobility Shift Assay**—Nuclear extracts from HepG2 cells were prepared as described previously (19). 33-bp oligonucleotides containing the SBE and CRE-like site in the Hp promoter 5′-GTTTTGTGTAAGAGGAATCTGGAATCTGATCAGGACAGA-3′ (forward) and 5′-CCTGATAGGTAAGAGGAATCTGGAATCTGATCAGGACAGA-3′ (reverse) were synthesized (Bioneer Corp., Daejeon, Korea) and used as probes. The oligonucleotide and its complementary oligonucleotide (2 nmol of each) were heated for 10 min in 10 μl of buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM EDTA and annealed by gradual cooling to room temperature. Double-stranded oligonucleotides were purified by polyacrylamide gel electrophoresis and end-labeled using [γ-32P]ATP (Bristol-Myers Squibb Co.) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA). For each binding reaction, 1 μg of poly(dI-dC), 12 μg of salmon sperm DNA, 5 μg of nuclear extract, and 100,000 cpm labeled probe were incubated for 30 min at room temperature. For competitive reactions, a 30-fold molar excess of unlabeled double-stranded oligonucleotides was incubated with the nuclear extracts for 30 min before the addition of radiolabeled probe. For supershift assay, 4 μg of each anti-STAT3, anti-HIF-1α, anti-p300, and anti-CRE-binding protein (CREB) antibodies (Santa Cruz Biotechnology) were added to the binding reactions before the addition of radiolabeled probe. The binding products were separated by 6% nondenaturing polyacrylamide
gel electrophoresis and detected by autoradiography of a dried gel.

**ChIP Assay**—A ChIP assay was performed as described previously (20). Cell lysates were sonicated on ice to shear DNA to 200- and 1000-bp fragments. Chromatin solutions were precleared using protein A-agarose/salmon sperm DNA (50% slurry; Upstate Biotechnology) with agitation for 30 min at 4 °C. The precleared supernatants were incubated with anti-STAT3, anti-HIF-1α/H9251, anti-CREB, or anti-p300/CBP antibody (Santa Cruz Biotechnology) overnight at 4 °C with rotation. Immune complexes were pelleted by the addition of protein A-agarose/salmon sperm DNA. Chromatin was eluted from antibody with a solution of 1% SDS and 0.1 M NaHCO3. NaCl (0.2 M final concentration) was added to the eluates, and the solution was incubated at 65 °C for 4 h to reverse DNA-protein cross-links.

After proteins were digested with proteinase K (Applied Biological Materials, British Columbia, Canada), DNA was purified by phenol/chloroform extraction and ethanol precipitation. The region containing the SBE and CRE-like site in Hp promoter/H11002-183/H11001-26 was amplified by PCR using the following primers: 5′-ACTGGTACCACAAGAAAATCAGTGTAAGAGCA-3′ (forward) and 5′-GTTGGTGCTTGGCTGGAAGAGCAGTG-3′ (reverse). The PCR products were analyzed on 1% agarose gels.

Reverse ChIP assay was performed to confirm the association of HIF-1α, STAT3, and p300/CBP. After the first immunoprecipitation by an antibody, the eluted chromatin was reprecipitated by a secondary antibody. DNA was extracted, and PCR amplification was carried out using the same primers.

**Statistical Analysis**—Data are expressed as means ± S.D. Statistical analyses were performed using Student’s t test. A p value of <0.05 was considered statistically significant.

**RESULTS**

**Involvement of HIF-1α in Hp gene expression**—In HepG2 cells, IL-6 treatment increased the HIF-1α protein level, as well as STAT3 phosphorylation and Hp expression (Fig. 1A). When HIF-1α was overexpressed by transient transfection of the gene, Hp expression was increased (Fig. 1, B and C). However, the levels of C/EBPβ and phosphorylated STAT3, which are known as major transcription factors for Hp gene expression, remained unchanged (Fig. 1B). When HIF-1α was knocked down by specific siRNA transfection, Hp expression was significantly decreased at both the mRNA and protein levels, without a change in STAT3 phosphorylation (Fig. 1, D and E). To determine whether the action of HIF-1α on IL-6-induced STAT3 target genes is general, gene expression of fibrinogen was tested. However, neither overexpressing nor silencing HIF-1α affected fibrinogen expression, in contrast to Hp expression (Fig. 1, B–E). These findings suggest that HIF-1α specifically participates in Hp gene activation, without increasing C/EBPβ and active STAT3.

**Enhancement of Hp Expression in Hypoxia**—Hypoxia increases the protein stability of HIF-1α through preventing...
von Hippel-Lindau protein-mediated degradation (21). HepG2 cells were incubated in a hypoxic chamber for 5 h in glucose-free DMEM with or without 10 ng/ml rIL-6. HIF-1α, phospho-STAT3 (PY-STAT3), STAT3, Hp, and β-actin were detected by Western blotting (A), and the Hp transcript was analyzed by RT-PCR (B). C–F, HepG2 cells were transfected with control siRNA (siControl) or HIF-1α siRNA (siHIF-1α). After 24 h, the cells were exposed to hypoxia for 5 h in the absence (C and D) or presence (D and F) of 10 ng/ml IL-6. The levels of proteins and mRNAs of interest in the HIF-1α-silenced cells were examined by Western blotting and RT-PCR, respectively. In the Western blots (A, C, and D), the band intensity was measured, and each protein level was normalized to the corresponding β-actin level. The results are expressed as relative quantity to each control (first lane of each blot). All experiments were carried out three times at least, and the results were similar.

Identification of Target Promoter Regulatory Regions Required for HIF-1α-induced Hp Gene Activation—A plasmid containing the 5′-flanking region of the human Hp gene (−994 to +26) and a luciferase reporter gene was constructed. When the plasmid and HIF-1α gene were cotransfected into HepG2 hepatoma cells, luciferase activity increased ~2-fold in the presence of HIF-1α. The Hp gene activation by HIF-1α was synergistic with 5 ng/ml rIL-6 treatment (Fig. 3B). To determine the promoter region responsive to HIF-1α in Hp gene activation, four truncated plasmid constructs were prepared by serial 5′-deletion of the Hp promoter and cotransfected with HIF-1α cDNA. When cells were transfected with the deletion construct containing region −99 to +26, the HIF-1α-increased luciferase activity was not detected (Fig. 3C). These results indicate that a small promoter region between positions −183 and −100 is required for HIF-1α-induced Hp gene activation.

Requirement for Both the Proximal SBE and Adjacent CRE-like Site for HIF-1α-induced Hp Expression—According to a computer-assisted analysis of the active −183/−100 regions of the Hp promoter, these regions contain two potential cis-acting elements, a SBE and a CRE-like site located 7 bp from the SBE (Fig. 4A). However, there was no hypoxia-response element for HIF-1α binding. To determine whether the CRE-like site and SBE are required for the response to HIF-1α, the sites were mutated (Fig. 4A). When WT or mutant constructs were
cotransfected with the HIF-1α gene, neither the SBE nor CRE mutants were responsive to HIF-1α. In contrast, wild-type promoter activity was increased 2-fold (Fig. 4B). Interestingly, IL-6 responsiveness was partially suppressed by CRE mutation and completely inhibited by SBE mutation (Fig. 4C). To further confirm the requirement of the SBE/CRE, EMSA was performed using a WT probe (−126 to −94) containing the SBE/CRE and three mutant probes (mutSBE, mutCRE, and mutSBE/mutCRE) (Fig. 5A). The WT probe largely bound to nuclear proteins extracted from HIF-1α-transfected cells. However, the binding activities of all three mutant probes were markedly reduced (Fig. 5B). Competition experiments with excess unlabeled probes also demonstrated that the mutant probes were inactive in binding (Fig. 5B). These results indicate that the two DNA motifs (SBE and CRE-like site) found in the Hp promoter are necessary for HIF-1α-stimulated Hp gene activation.

**Binding of STAT3, HIF-1α, and p300/CREB to the Promoter Sites Involving the SBE/CRE—**We examined whether STAT3 and CREB actually bind to the active promoter region described above. As shown in Fig. 6A, anti-STAT3 antibody attenuated
the formation of the binding complex, indicating that STAT3 is a component of the Hp promoter complex. However, CREB was not involved in the binding complex. Interestingly, HIF-1α was also a component of the binding complex in the SBE/CRE region (Fig. 6B). Because of the absence of an expected HIF-1α-binding element in the region, it appeared that HIF-1α binding might be indirect. The transcription coactivator p300/CBP associates with HIF-1α, as well as STAT3, in the formation of an active transcription complex for the transactivation of several genes (22, 23). Therefore, we examined the association of p300/CBP in the complex. Fig. 6B shows that the Hp promoter complex contains p300/CBP. Because an equal amount of normal rabbit IgG did not affect complex formation (Fig. 6C), the effect of the antibodies was thought to be specific. These results suggest that STAT3, HIF-1α, and p300/CBP are associated as a transcription complex that regulates HIF-1α-induced Hp expression in HepG2 hepatoma cells.

**HIF-1α Mediates Strong Binding of STAT3 to the Hp Promoter**—To determine whether STAT3 and HIF-1α interact mutually with the Hp promoter, a ChIP assay was carried out. The results showed that association of STAT3 with the Hp promoter was significantly increased by HIF-1α overexpression (Fig. 7B). Reverse ChIP data also showed that STAT3, HIF-1α, and p300/CBP simultaneously associated on the Hp promoter region containing the SBE/CRE (Fig. 7, C and D). When HIF-1α was specifically knocked down by siRNA transfection, STAT3 binding to the Hp promoter region was decreased (Fig. 7E). These findings suggest that STAT3, HIF-1α, and p300/CBP associate cooperatively and bind to the SBE/CRE in the Hp promoter as an active transcription complex and that HIF-1α may induce strong binding of STAT3 to the SBE and lead to high-level Hp expression.

**FIGURE 5.** HIF-1α augments the binding of nuclear proteins on an oligonucleotide probe containing the SBE/CRE. A, the sequences of oligonucleotide probes used in EMSA are shown. The WT probe contains sequences corresponding to positions –126 to –94 of the Hp promoter, including a SBE and a CRE-like site. The SBE and CRE-like site are boxed, and the mutated nucleotides are shown as **boldface**, _italicized_, and _underlined_ letters in the boxes (mutSBE and mutCRE). B, 32P-labeled WT and mutant probes were incubated with 5 μg of nuclear proteins extracted from vector- or HIF-1α-transfected HepG2 cells. For competition experiments, the nuclear extracts were preincubated with unlabeled probes at 30-fold molar excess. The complexes were resolved on 6% nondenaturing polyacrylamide gel and visualized by autoradiography. Similar results were obtained in three independent experiments, and the representative data are shown.

**FIGURE 6.** Binding of STAT3, HIF-1α, and p300/CBP to the –126/–94 region of the Hp promoter. Nuclear extracts were incubated with 4 μg of each antibody against STAT3 or CREB (A) or against HIF-1α or p300/CBP (B) or with 4 μg of normal rabbit IgG (C). After 30 min, the 32P-labeled WT probe was added to the each sample mixture and further incubated for 30 min. Note that the anti-STAT3, anti-HIF-1α, and anti-p300/CBP antibodies interrupted the binding of transcription factors to the probe, but the anti-CREB antibody did not affect the binding. All experiments were performed twice at least, and similar results were obtained.
To determine whether HIF-1α/H9251 can act STAT3-independently in haptoglobin expression, we carried out the experiment with high HIF-1α levels but low STAT3 activity. To induce this condition, auranofin (STAT3 inhibitor) (24) and IL-6 (HIF-1α inducer as well as STAT3 activator) were combined. When HepG2 cells were preincubated with auranofin before IL-6 treatment, IL-6-induced STAT3 phosphorylation was markedly inhibited, but HIF-1α remained at high levels. Under these conditions, Hp expression was attenuated (Fig. 8A). ChIP data indicated that HIF-1α binding to the Hp promoter was not increased in the presence of auranofin (Fig. 8B). These results confirm that HIF-1α does not act directly but assists in the strong binding of STAT3 to the Hp promoter for high transcriptional activity.

The reason why HIF-1α was not decreased by auranofin (Fig. 8A), although it is a target of active STAT3 (12, 13), is probably due to the increased protein stability of HIF-1α with an inhibited proteasome system. The possibility is supported by previous work demonstrating that the gold(I) compound auranofin increases the protein stability of Nrf2 (nuclear factor erythroid 2-related factor-2) and that another gold(III) compound inhibits proteasome activity (25, 26).

**DISCUSSION**

We have demonstrated, for the first time, that HIF-1α potentiates transcription of the Hp gene by assisting in the strong binding of STAT3 to the cis-acting SBE within the promoter and that the CRE-like site located near the SBE is required. The HIF-1α regulation seems to be specific for characteristic genes with the SBE and CRE located adjacent, such as the Hp gene, because fibrinogen expression was not regulated by HIF-1α (Fig. 1, B–E). Fibrinogen is also an acute phase protein induced by IL-6, and STAT3 has important roles in the gene expression, but the gene has no CRE around the active SBE (27).

Hp was up-regulated not only by HIF-1α overexpression by gene transfection but also by hypoxia. HepG2 cells incubated in a hypoxic chamber for 5 h in glucose-free medium had elevated levels of HIF-1α and Hp. Hypoxia in the presence of IL-6 synergistically induced Hp, as STAT3 phosphorylation was more enhanced (Fig. 2). This coincides with the result of others demonstrating that hepatic Hp expression is elevated under hypoxic conditions (28). However, a longer hypoxic stimulation, especially in the presence of glucose, decreased Hp expression, and a low level of active STAT3 was observed, in accordance with the low Hp level (data not shown). Results regarding STAT3 activation by hypoxia have been controversial. Choi et al. (29) reported that hypoxia inhibits phosphorylation of STAT3 via generation of reactive oxygen species in HepG2 hepatoma cells. On the other hand, the cooperative induction of phosphorylated STAT3 and HIF-1α was shown during hypoxia in a lung carcinoma cell line (30). Therefore, STAT3 activation under hypoxic conditions seems to be dependent on cell types or other factors induced by hypoxia such as reactive oxygen species and SOCS proteins. Based on findings in our study, we...
believe that Hp can be maximally expressed in hypoxic environments with STAT3 activation.

Glucocorticoid hormone acts synergistically with IL-6 in human hepatic Hp expression (31). Because no glucocorticoid-responsive element is found in the human Hp gene, it is likely that glucocorticoid-induced factors (rather than the activated hormone receptor) are associated in Hp transcription. To investigate whether HIF-1α contributes to the glucocorticoid-regulated Hp expression as a partner protein of STAT3, HepG2 cells were treated with a combination of IL-6 and dexamethasone. The combined treatment of IL-6 and dexamethasone resulted in a much greater increase in Hp expression and induction of HIF-1α. Therefore, we believe that Hp can be maximally expressed in hypoxic environments with STAT3 activation.

Oliviero and Cortese (11) identified three regions (A, B, and C) responsible for IL-6 signaling in the human Hp gene promoter, which are localized within −186 bp of the proximal 5’-flanking region. Regions A and C are binding sites for C/EBPβ, and region B contains the SBE. The region involved in HIF-1α activation of Hp gene transcription identified here corresponds to promoter region B.

Our results indicate that two DNA elements (SBE and CRE-like site), located adjacent at positions −120 to −97 within the Hp promoter, are critical for HIF-1α-potentiated Hp gene activation and the binding of trans-acting factors (Figs. 4 and 5). However, our results show that CREB does not participate in HIF-1α-enhanced Hp expression (Fig. 6). Interestingly, despite the absence of a consensus HIF-1α-binding element, HIF-1α existed as a component of an active transcription complex bound on the target SBE/CRE (Figs. 6 and 7). It has been reported that HIF-1α associates with activated STAT3 and binds to the VEGF promoter and that the proteins form a transcription complex with p300/CBP for maximum expression of the target gene (14, 15). Our data also show that HIF-1α, STAT3, and p300/CBP are associated and bind simultaneously to the Hp promoter. Furthermore, knockdown of HIF-1α attenuated the binding of STAT3 to the SBE (Fig. 7). Thus, it appears that HIF-1α associated with STAT3 strengthens the binding of STAT3 to the SBE and that the adjacent CRE-like site plays an important role. Although it is still unclear whether HIF-1α directly binds the CRE-like site, HIF-1α is thought to be a candidate for the partner protein for STAT3 in Hp gene expression.

Several genes, such as STAT3, junB, and IRF1 (interferon regulatory factor-1), have a target IL-6RE in their promoters (17, 18, 32, 33). The IL-6RE contains both a SBE and an adjacent CRE, in which the two cooperative DNA motifs are spaced 4 or 5 bp apart. The STAT3, junB, and IRF1 IL-6REs consist of an atypical SBE with a consensus CRE (TGACGTC), an atypical SBE with an atypical CRE, and a consensus SBE (TTN6, AA) with an atypical CRE, respectively. Active phosphorylated STAT binds to the IL-6RE in combination with other DNA-binding proteins, which affect the specificity and affinity of the complex (34–37). Among the unidentified joining proteins, HIF-1α may be a component of the active transcription complex. A study will be needed to confirm whether HIF-1α also participates in signal transduction of STAT3, junB, and IRF1 containing the cooperative SBE and CRE in their promoter regions.

The plasma concentration of Hp is elevated 2–4-fold via signaling of IL-6 produced at high levels during inflammatory responses (38). Our data indicate that IL-6 stimulates not only STAT3 activation but also HIF-1α induction in hepatic cells
Cooperation of HIF-1α and STAT3 in Haptoglobin Expression

(Fig. 1A). Considered together with these findings, it appears that Hp can be effectively expressed through the cooperating actions of STAT3 and HIF-1α in the modulation of inflammation. Hp levels are also increased in patients with malignant tumors (39). Because a number of solid tumors are exposed to hypoxic environments, HIF-1α is preserved at high levels through increased protein stabilization (21). STAT3 is also constitutively activated in various malignant tumors (40, 41). Based on the results of our study, it is possible that, in the tumor microenvironment containing abundant active STAT3 and HIF-1α, Hp can be effectively expressed via the synergistic actions of the two transcription factors and contributes to the enhanced levels in cancer. In fact, we (43) and others (42) have reported the Hp production in reactive astrocytes after transient forebrain ischemia and in human glioblastoma cells, respectively.

Although the biologic functions of Hp in tumor environments still remain to be solved, Hp is thought to participate in the formation of new blood vessels and vascular remodeling due to its pro-angiogenic and cell-migrating properties (6–9). A very recent study demonstrated that Hp plays an important role in the migration of human glioblastoma cells (42), an aggressive brain tumor, and HIF-1α, as well as STAT3, is required for the tumor development (41, 44, 45). Furthermore, in a tumor xenograft model, mouse-implanted Hp-overexpressing glioma cells showed increased tumor growth and a decreased survival rate (42). Our study supports the possibility that Hp may be up-regulated locally in cancer environments providing active STAT3 and hypoxia and participate in angiogenesis and cell migration, which promote tumor growth and invasion.

Acknowledgment—We thank Professor Kyu-Won Kim (Angiogenesis Gene Bank, Angiogenesis Research Laboratory) for the gift of HIF-1α cDNA.

REFERENCES

1. Baumann, H., and Gauldie, J. (1990) Mol. Biol. Med. 7, 147–159
2. Baumann, H., and Gauldie, J. (1994) ImmunoL. Today 15, 74–80
3. Nielsen, M. J., and Moestrup, S. K. (2009) Blood 114, 764–771
4. Lim, S. K., Kim, H., Lim, S. K., bin Ali, A., Lim, Y. K., Wang, Y., Chong, S. M., Costantini, F., and Baumann, H. (1998) Blood 92, 1870–1877
5. Lim, Y. K., Jenner, A., Ali, A. B., Wang, Y., Hsu, S. I., Chong, S. M., Baumann, H., Halliwell, B., and Lim, S. K. (2000) Kidney Int. 58, 1033–1044
6. Cid, M. C., Grant, D. S., Hoffman, G. S., Auerbach, R., Fauci, A. S., and Bakale, N. (1993) Clin. Invest. 91, 977–985
7. de Kleijn, D. P., Smeets, M. B., Kemmeren, P. P., Lim, S. K., Van Middelaar, B. J., Velena, E., Schoneveld, A., Pasternak, G., and Borst, C. (2002) FASEB J. 16, 1123–1125
8. Lohr, N. L., Wartliier, D. C., Chilain, W. M., and Weihrauch, D. (2005) Am. J. Physiol. Heart Circ. Physiol. 288, H1389–H1395
9. Wenger, R. H., Rolf, A., Martin, H. B., Bauer, C., and Gassmann, M. (1995) J. Biol. Chem. 270, 27865–27870
10. Koim, H., Oh, M. K., Park, S. J., Choi, J. S., Oh, M. K., and Kim, I. S. (2007) Immunology 122, 607–614
11. Yu, H., and Jove, R. (2004) Cancer Res. 64, 10478–10486
12. Duan, H. O., and Simpson-Haidar, P. J. (2003) J. Biol. Chem. 278, 41270–41281
13. Wang, Y., Kinzie, E., Berger, F. G., Lim, S. K., and Baumann, H. (2001) Redox Rep. 6, 379–385
14. Ahmed, N., Barker, G., Oliva, K. T., Hoffmann, P., Riley, C., Reeve, S., Smith, A. L., Kemp, B. E., Quinn, M. A., and Rice, G. E. (2004) Br. J. Cancer 91, 129–140
15. Schaeffer, T. S., Sanders, L. K., and Nathans, D. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 9097–9101
16. Xu, X., Sun, Y. L., and Hoey, T. (1996) Science 273, 794–797
17. Wang, Y., Kinzie, E., Berger, F. G., Lim, S. K., and Baumann, H. (2001) J. Biol. Chem. 276, 4089–4096