Dmitriy Lukashev, Charles Caldwell, Akio Ohta, Pearl Chen, and Michail Sitkovsky‡

From the Laboratory of Immunology, NIAID, National Institutes of Health, Bethesda, Maryland 20892

Cell adaptation to hypoxia is partially accomplished by hypoxia-inducible transcription factor-1 (HIF-1). Here we report the hypoxia-independent up-regulation of HIF-1α subunit in antigen receptor-activated T cells. This is explained by a selective up-regulation of alternatively spliced mRNA isoform I.1 that encodes the HIF-1α protein without the first 12 N-terminal amino acids. We show that both short (I.1) and long (I.2) HIF-1α isoforms display similar DNA binding and transcriptional activities. Major differences were observed between these two HIF-1α isoforms in their expression patterns with respect to the resting and activated T lymphocytes in hypoxic and normoxic conditions. The T cell antigen receptor (TCR)-triggered activation of normal ex vivo T cells and differentiated T cells results in up-regulation of expression of I.1 isoform of HIF-1α mRNA without an effect on constitutive I.2 HIF-1α mRNA expression. The accumulation of I.1 HIF-1α mRNA isoform in T lymphocytes is also demonstrated during cytokine-mediated inflammation in vivo, suggesting a physiological role of short HIF-1α isoform in activated lymphocytes. The TCR-triggered, protein kinase C and Ca²⁺/calcineurin-mediated HIF-1α I.1 mRNA induction is protein synthesis-independent, suggesting that the HIF-1α I.1 gene is expressed as an immediate early response gene. Therefore, these data predict a different physiological role of short and long isoforms of HIF-1α in resting and activated cells.

Immune cells are exposed to different oxygen tensions, including hypoxia, as they develop, migrate, and function in primary, secondary, and tertiary lymphoid organs with different infrastructure, vasculature, and oxygen supply (1, 2). Hypoxic extracellular environments were demonstrated in some normal tissues (3, 4) and during chronic inflammatory and malignant diseases (5–10). The mechanisms of lymphocyte adaptation to hypoxia are likely to exist under such conditions.

Cell adaptation to hypoxia is partially accomplished by the transcriptional activity of hypoxia-inducible factor-1 (HIF-1).¹ HIF-1 is a basic helix-loop-helix/Per-ARNT-Sim protein consisting of HIF-1α and HIF-1β subunits (11, 12). The HIF-1β subunit is also known as aryl hydrocarbon receptor nuclear translocator (ARNT) and serves as a heterodimerization partner for other transcription factors (13–17). HIF-1 activates the transcription of genes required for glucose metabolism, erythropoiesis, vascularization, and cell proliferation by binding to cis-acting hypoxia response element (HRE) (18–22). The HIF-1α subunit may also affect cell metabolism and signaling by its ability to directly interact with other proteins such as p53 (23). Multiple roles of HIF-1α as transcriptional factor and in protein-protein interactions complicate the understanding of its role in vivo. Possible clues are expected to be provided by studies of regulation of HIF-1α mRNA and protein expression.

Oxygen-sensing mechanisms and the subsequent regulation of HIF-1 expression are the subject of intensive investigations. It was shown that HIF-1α, but not HIF-1β, expression is significantly enhanced by hypoxia (12, 24, 25). It is believed that the regulation of HIF-1α expression occurs mostly on post-translational level (19). HIF-1α mRNA is constitutively expressed in tissue culture cells independent of oxygen tensions (26, 27), but its expression is induced by hypoxia or ischemia in vivo (28–30). Protein stability plays most important role in control of HIF-1α expression. At high oxygen tensions, HIF-1α is targeted for destruction by an E3 ubiquitin ligase containing the von Hippel-Lindau tumor suppressor protein (pVHL) (31, 32). According to a current model, pVHL binds to the oxygen-dependent degradation domain located in the central region of HIF-1α (33) that results in a subsequent degradation of HIF-1α through the ubiquitin-proteasome pathway (34, 35).

We were prompted to revisit this model by recent demonstrations of oxygen tension-independent induction of HIF-1α by hormones (36) and proinflammatory cytokines (37) as well as by our own studies of HIF-1α expression in activated T lymphocytes.² It was also important to investigate the possibility of differential expression of two mouse HIF-1α mRNA isoforms, which contain two alternative first exons named I.1 and I.2 (38, 39). The I.1 mRNA encodes a protein, which is expected to be 12 N-terminal amino acid residues shorter than HIF-1α I.2 mRNA-encoded protein, although no difference in functions of these HIF-1α isoforms has yet been reported (40). No corresponding human isoform has been found so far. The ratios of these two mRNA isoforms in cells and patterns of expression of HIF-1α I.1 mRNA in resting versus activated versus differentiated cells are not known. HIF-1α I.2 mRNA is constitutively expressed like a housekeeping gene in all tissues in an oxygen tension-independent manner, while I.1 mRNA has a tissue-specific expression (39).

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‡ To whom correspondence should be addressed: Bldg. 10, Rm. 11N311, Laboratory of Immunology, NIAID, National Institutes of Health, Bethesda, MD 20892. Tel.: 301-496-5495; Fax: 301-480-7352; E-mail: mvsitkov@helix.nih.gov.

¹ The abbreviations used are: HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor; iNOS, inducible nitric oxide synthase; HRE, hypoxia response element; ARNT aryl hydrocarbon receptor nuclear translocator; pVHL, von Hippel-Lindau protein; RT, reverse transcription; TCR, T cell antigen receptor; ConA, concanavalin A; mAb, monoclonal antibody; MES, 4-morpholineethanesulfonic acid; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-[hydroxyethyl]propane-1,3-diol.

² C. Caldwell and M. Sitkovsky, manuscript in preparation.
Thus, studies of expression of these two HIF-1α mRNA isoforms may allow to us to distinguish possible separate roles of HIF-1α as a transcription factor and in protein-protein interactions provided that the expression of short and long forms of HIF-1α is differentially regulated. Accordingly, in this study we asked whether HIF-1α mRNA is indeed constitutively expressed in ex vivo naive T cells, ex vivo activated T cells, differentiated T cells, and resting versus activated T cells by taking advantage of the possibilities provided by quantitative competitive RT-PCR.

We report here that TCR-triggered activation of T lymphocytes results in up-regulation of the I.1 isoform of HIF-1α mRNA without an effect on 1.2 mRNA expression. The expression of I.1 HIF-1α mRNA follows a pattern of immediate early response genes. These observations suggest differential regulation and functions of these two isoforms of HIF-1α proteins in resting and activated cells.

EXPERIMENTAL PROCEDURES

Cells—Mouse 2B4 T helper hybridoma cells and mouse splenocytes were maintained in RPMI 1640 (Biofluids, Rockville, MD), supplemented with 5% dialyzed fetal calf serum (heat-inactivated) and 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 1 mM HEPES, nonessential amino acids (RP5), and 50 μM 2-mercaptoethanol (complete RPMI). NIH 3T3 fibroblasts were cultured in AMEM supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Single cell splenocyte suspensions were prepared from adult mouse spleens. T cells were isolated using a mixture of anti-CD4 and anti-CD8 microbeads to positively select cells on an AutoMACs (Miltenyi Biotec, Auburn, CA) using the manufacturer’s protocol. Isolated cells were incubated in complete RPMI 1640. Unless otherwise indicated, 50 μM 2-mercaptoethanol was added into cell cultures conducted at 20% oxygen tension as indicated in the figure legends. Th1 and Th2 cells were generated from wild type T cells by TCR and IFN-γ stimulation using 2-fold dilutions of mimic DNA. The Th1 mimic CDNA was 33 bp shorter than wild type HIF-1α CDNA (Fig. 1B). HIF-1α mRNA was determined by competitive RT-PCR for determination of either I.1 or 1.2 mRNA concentrations in cellular extracts. VEGF mimic mRNA was made by PCR on total mouse cDNA using primers TTTTTTGAATTCTTGAGTATAAGCGAAGTACCTGGTACAATGAG and TTTTTTGGATCCACGCTTTTCCAGAATTC (Promega, Madison, WI) and cloned into in pGEM-3Zf(+) (Promega, Madison, WI) by EcoRI-BamHI sites. Mimic cDNA for INOS was created from total mouse cDNA by PCR using primers AATAATGAATCTCATACAGACCAAGGACGCACGACGACG and AATAATGATCCGGCTCTCCAGAGGCTAG (G. 455-bp PCR product was digested with EcoRI, BamHI, and HindIII and cloned into in pGEM-3Zf(+) (Promega, Madison, WI) by EcoRI-BamHI sites.

Construction of Mimic for Competitive RT-PCR—The EcoRI-PstI fragment of human HIF-1α cDNA (GenBank™ number U22431), which has high homology with mouse HIF-1α (GenBank™ number AF003695) was subcloned into pGEM-3Zf(+) (Promega, Madison, WI) and restriction enzyme (XhoI) digestion. For deletion of the internal region from wild type cDNA, PCR was performed using M13 reverse primer and SpeI containing primer GAAGACTTGTCCGACGCTTCACCAAAC. PCR product was digested with PstI and SpeI and was inserted instead of the SpeI-PstI fragment in the HIF-1α EcoRI-PstI fragment. The resulting HIF-1α mimic CDNA was 33 bp shorter than wild type HIF-1α CDNA (Fig. 1B). I.1 (GenBank™ number Y09056) and 1.2 (GenBank™ number Y13656) mimic cDNA were constructed by PCR using TTTCTGGGCGCAACTGTTATTAACGACGATCCTTGGAATGGTATAAGCGAAGTACCTGGTACAATGAG and TTTTTTGGATCCACGCTTTTCCAGAATTC (Promega, Madison, WI) by EcoRI-BamHI sites. Mimic cDNA for INOS was created from total mouse cDNA by PCR using primers AATAATGAATCTCATACAGACCAAGGACGCACGACGACG and AATAATGATCCGGCTCTCCAGAGGCTAG (G. 455-bp PCR product was digested with EcoRI, BamHI, and HindIII and cloned into in pGEM-3Zf(+) (Promega, Madison, WI) by EcoRI-BamHI sites.

Determination of HIF-1α mRNA Expression by Competitive RT-PCR—RNA corresponding mimic cDNA was synthesized with T7 RNA polymerase (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s instructions. Transcription product was analyzed in 8% PAGE, and an 850-base nondegraded RNA band was observed. Optical dichroism of RNA product was measured at 260 nm, and 1 μg of mimic RNA was subjected to reverse transcription in the corresponding cDNA reactions for each sample (as for cDNA from wild type CD4+ T cells in vitro according to Ref. 41, and they were kindly provided by Dr. J. Hu-Li (NIAMD, National Institutes of Health). Activation of T Cells—The TCR-activated 2B4 cells were incubated (2 × 10⁶ cells/ml) in 96-well plates (Costar, Corning, NY) precoated with 10 μg/ml anti-TCR (clone H57-597; Pharmingen, La Jolla, CA). Studies of the biochemical pathways involved in HIF-1α mRNA regulation were performed after incubation of cells with 15 nM phorbol 12-myristate 13-acetate (Calbiochem) and/or 300 nM ionomycin (Calbiochem). Inhibitors were added as indicated: cycloheximide (Sigma) (50 μg/ml), actinomycin D (Bioulom, Plymouth Meeting, PA) (5 μg/ml), cyclosporin A (Bioulom, Plymouth Meeting, PA) (1 μg/ml), and K252b (20 μM). Mouse T cells were activated by plate-bound (5 μg/ml) anti-CD3 mAb (clone 145-2C11; Pharmingen, La Jolla, CA) and 10 μg/ml anti-CD28 mAb (clone 37.51; Pharmingen) for 36 h as described earlier (42). Activation of T Cells in Vivo—Female C57BL/6 mice were injected intravenously with 20 mg/kg concanavalin A (type IV; Sigma) dissolved in sterile phosphate-buffered saline. After 6 h, tissue samples were taken, and spleen T cells were isolated using AutoMACS as described above. Total RNA was isolated using the RNA STAT-60 kit (Tel-Test, Friendswood, TX) according to the manufacturer’s protocol. Measurements of cytokine levels (tumor necrosis factor-α and interferon-γ) in the sera were determined using enzyme-linked immunosorbent assay kits obtained from R&D systems (Minneapolis, MN) according to the manufacturer’s instructions. Western Blot Analysis—Cells were centrifuged and resuspended in 2× sample buffer (Novex) with 4% 2-mercaptoethanol followed by 30-s ultrasound treatment. Samples were boiled for 5 min and loaded to 7% SDS-PAGE. An immunoblot assay was performed as described (12) except that 1:200 diluted antibodies (Transduction Laboratories, Lexington, KY) were used.

RNA Isolation and Northern Blot Analysis—Total RNA was extracted from 10⁶ to 10⁷ cells using RNA STAT-60 kit (Tel-Test, Friendswood, TX) according to the manufacturer’s protocol. Northern blotting was performed following the general procedure (formaldehyde gel) (43) using 1 μg of messenger RNA purified with the Oligotex mRNA Mini Kit (Qiagen, Chatsworth, CA) per lane. HIF-1α probe was synthesized using a 380-bp HIF-1α cDNA fragment. The glyceraldehyde-3-phosphate dehydrogenase probe was purchased from CLONTECH (Palo Alto, CA).
HIF-1α, and human ARNT mRNAs, respectively, by T7 RNA polymerase (Roche Molecular Biochemicals), which were subsequently used for translation in vitro using rabbit reticulocyte lysate system (Promega, Madison, WI) with or without [35S]Met (Amersham Biosciences, Inc.). After that, 5 μl of unlabeled in vitro synthesized ARNT and either one of the HIF-1α isoforms were incubated for 30 min at room temperature before the addition of 10⁶ cpm of 32P-labeled ([γ-32P]ATP for 5′ labeling of oligonucleotide) was purchased from Amersham Biosciences) double-stranded oligodeoxyribonucleotide AGCT-TGCCCTACGTGCTGTCTCAG, corresponding to HRE from the sham Biosciences) double-stranded oligodeoxyribonucleotide AGCT-TGCCCTACGTGCTGTCTCAG, corresponding to HRE from the human erythropoietin gene enhancer, and binding buffer (20 mM Tris-HCl pH 7.5, 50% glycerol, 0.1 mM KCl, 0.2 mM EDTA, 2.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4) to a final volume of 40 μl. The same but unlabeled double-stranded oligonucleotide was used as a competitor in 20-fold excess. After 20 min of incubation at 4 °C, the samples were resolved using 5% non-denaturing gel with 0.5× TBE.

**Transient Expression Assay**—Expression vector containing the HIF-1α isoform was prepared by cloning HIF-1α DNA containing 45-bp 5′-untranslated region in pCEP4 plasmid (CLONTECH, Palo Alto, CA). An expression construct of the I.1 isoform was created by deletion of 36 bp corresponding to amino acid residues 1–12 from I.2 cDNA. Human HIF-1α expressing vector pCEP4/HIF-1α-T7 was kindly provided by Dr. G. Semenza (Johns Hopkins University, Baltimore, MD), and plasmid pBBS/Neo/M1 containing human ARNT was a kind gift from Dr. O. Hankinson. Reporter plasmid HRE-Luc, containing three copies of iNOS promoter-derived HRE, was kindly provided by Dr. M. Blagosklonny (NCI, National Institutes of Health). 5 × 10⁴ NIH 3T3 cells/well were plated in 24-well plates (Costar, Corning, NY) the day before transfection. Cells were transfected in triplicate using LipofectAMINE PLUS reagent (Life Technologies) in the same medium according to the manufacturer’s protocol. The following amounts of plasmids were used: 0.1 μg of HRE-Luc, 0.2–0.5 μg of one of the HIF-1α-expressing vectors, 0.2–0.5 μg of pBBS/Neo/M1. The total amount of DNA/well was adjusted to 1 μg with pGEM-3Zf (+) (Promega, Madison, WI). Sixteen hours after transfection, fresh medium was added, and cells were grown for an additional 24 h at either 1 or 20% O2. Luciferase activity was determined using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer’s protocol.

**RESULTS**

**Up-regulation of Expression of HIF-1α mRNA in Antigen Receptor-activated T Cells**—Our studies of T cell functions under hypoxic conditions revealed HIF-1α protein up-regulation in TCR-triggered activated T lymphocytes under normoxic conditions (20% O2). T lymphocytes are not unique in functions of immediate early genes in T cell activation. We undertook detailed studies of HIF-1α activation in activated T cells, since HIF-1α has important transcriptional activities and affects important regulatory proteins. Up-regulation of HIF-1α protein and HIF-1α mRNA in activated versus nonactivated T cells is demonstrated by Western and Northern blot analysis of extracts from 2B4 T helper hybridoma cells after their incubation with anti-TCR mAb at nonhypoxic conditions at 20% oxygen (Fig. 1A). Increased amounts of HIF-1α mRNA in activated T cells was unexpected, since HIF-1α regulation was known to be controlled on a post-translational level (19). Further detailed mRNA expression studies by Northern blot analysis of HIF-1α mRNA in T lymphocytes were limited by the requirement of the large numbers of cells and inadequate quantitative measurement of HIF-1α mRNA increase by Northern blot analysis of mRNA isoforms. This prompted the development of quantitative competitive RT-PCR assay using the mimic corresponding to the 3′ region of HIF-1α mRNA (Fig. 1B). We determined that HIF-1α mRNA expression is greater than 5-fold higher in TCR-stimulated T cells cultured at 1% O2 as compared with nonstimulated cells, with a 15-fold increase in HIF-1α mRNA in cells activated in normoxic conditions, at 20% O2 (Fig. 1C). Direct measurements established that there were lower levels of HIF-1α mRNA in ex vivo CD4+ naive T cells than in Th1 and Th2 T cells obtained after their differentiation in vitro (data not shown). Therefore, TCR-mediated activation, but not hypoxia, leads to HIF-1α mRNA expression up-regulation in T cells.

Incubation of T cells with TCR-cross-linking reagents triggers a complex cascade of events such as inositol-trisphosphate signaling, increases in intracellular Ca⁡²⁺, Ca⁡²⁺/CaM-dependent phosphatase, and calcineurin and protein kinase C activation (46). To test whether these pathways may be implicated in HIF-1α mRNA up-regulation, we tested the effects of protein kinase C activators and Ca²⁺ ionophore. It appears that these pathways are responsible, in part, for the observed increases in HIF-1α mRNA expression. Protein kinase C activator phorbol 12-myristate 13-acetate and Ca²⁺ ionophore ionomycin were able to mimic the effects of TCR cross-linking on HIF-1α mRNA expression in T cells (Fig. 1D). Additionally, inhibition of TCR-triggered HIF-1α mRNA up-regulation by inhibitors of calcineurin (cyclosporin A) and protein kinase C (R252b) supports such an interpretation (data not shown).

**HIF-1α Is an Immediate Early Response Gene in Antigen Receptor-activated T Cells**—The HIF-1α mRNA up-regulation at nonhypoxic conditions by TCR cross-linking in T cells (Fig. 1) prompted us to investigate the mechanisms of HIF-1α up-regulation using competitive RT-PCR in time course studies (Fig. 2A).

The expression of HIF-1α mRNA increased within 1 h of TCR cross-linking, with the fastest accumulation between 3 and 6 h and plateauing after 6 h of activation (Fig. 2A). These data both confirm that HIF-1α mRNA is up-regulated upon TCR-triggered activation and suggest that such rapid induction of HIF-1α mRNA may indicate the involvement of HIF-1 in functions of immediate early genes in T cell activation.

The rapid (from minutes up to 4 hours) and de novo protein synthesis-independent increase in mRNA expression is considered to be a hallmark of immediate early genes (47). To test whether HIF-1α expression follows the pattern of immediate early response genes, we measured the HIF-1α mRNA expression in activated T cells in the presence of an inhibitor of protein synthesis, cycloheximide. Cycloheximide did not affect up-regulation of HIF-1α mRNA in activated 2B4 cells (Fig. 2B), indicating that increases in HIF-1α mRNA expression are accomplished by preexisting proteins. To distinguish between HIF-1α mRNA stabilization versus the de novo HIF-1α mRNA synthesis as the mechanisms observed in HIF-1α mRNA up-regulation in TCR-activated T cells, we utilized actinomycin D, an RNA polymerase inhibitor (Fig. 2C). Actinomycin D does inhibit HIF-1α mRNA up-regulation, suggesting that transcription of the HIF-1α gene, rather than HIF-1α mRNA stabilization, is required for the effects of TCR cross-linking on HIF-1α mRNA expression in activated T cells. We therefore conclude that HIF-1α is an immediate early response gene in antigen receptor-activated T cells, since its transcription is rapidly up-regulated upon TCR-triggered T cell activation by preexisting cellular factors.

**Differential Up-regulation of Expression of I.1 and I.2 mRNA Isoforms of Hypoxia-inducible Factor-1α in Antigen Receptor-activated Lymphocytes in Vitro and in Vivo**—Next, we attempted to determine whether the up-regulation of HIF-1α mRNA in T cells is due to up-regulation of HIF-1α I.1 or I.2 mRNA or both isoforms. It was shown that the mouse genome contains two alternative exons for HIF-1α mRNA, I.1
and I.2 (38). While I.2 HIF-1α mRNA is expressed like a housekeeping gene, the expression of I.1 HIF-1α mRNA has tissue specificity and can be found in spleen and thymus (39).

To test whether both or one HIF-1α mRNA is expressed in TCR-activated T cells, we performed RT-PCR on RNA samples from T cells and 2B4 hybridoma cells using primers specific for I.1 and I.2 exons. The I.2 HIF-1α mRNA isoform is expressed in both nonactivated and activated T cells in both hypoxic and nonhypoxic conditions (Fig. 3A). In contrast, only I.1 mRNA was up-regulated by T cell activation. The I.1 mRNA was not observed in nonactivated peripheral T cells or T cell hybridomas (Fig. 3A).

To enable simultaneous measurements of I.1 and I.2 HIF-1α mRNA isoforms in further studies, a competitive RT-PCR for I.1 and I.2 HIF-1α mRNAs has been developed (Fig. 3B) by constructing appropriate mimics. It is shown that the I.1 HIF-1α mRNA isoform is indeed up-regulated by T cell activation but not hypoxia (Fig. 3C), while the I.2 exon is expressed constitutively (Fig. 3D). Therefore, the differential regulation of these two isoforms in T cells and the functioning of the activation-dependent mechanism of up-regulation of I.1 HIF-1α mRNA in T cells were established.

To further support this theory based on in vitro experiments, we demonstrated the activation-dependent I.1 HIF-1α mRNA expression in vivo. This was accomplished with a ConA-induced model of inflammation in vivo, where the proinflammatory cytokine secretion is dependent on activation of T and NKT cells (48). It is shown that intravenous...
injection of ConA results in a “cytokine storm,” as illustrated by strong up-regulation of levels of interferon-γ and tumor necrosis factor-α in serum (Fig. 3E). It is also shown (Fig. 3E), that I.1 mRNA expression is increased about 20-fold at in vivo activated spleen T cells from ConA-injected animals. Similar increases were also found, in lymphocytes from lymph nodes and thymus (data not shown). Thus, these data provide evidence that T cell receptor stimulation up-regulates the HIF-1α I.1 mRNA isoform both in vitro and at in vivo conditions.

Long and Short HIF-1α Isoforms Possess Similar DNA Binding and Transcriptional Activities—It was interesting to continue our studies by analyzing the expression patterns and properties of “long” and “short” HIF-1α isoforms on protein level. There are, however, limitations in our ability to observe differential expression of these two proteins by Western blots. Indeed, the observed differential accumulation of I.1 HIF-1α mRNA suggests that I.1 mRNA would be translated (40) into the HIF-1α “short” protein isoform lacking 12 amino acid residues at the N end as compared with the long I.2 isoform that contains 836 amino acid residues (49) (Fig. 4A). We were unable to discern between these two protein isoforms because of the small size difference between these proteins (data not shown). This precluded comparative studies of HIF-1α isoforms on protein levels by immunoblotting, although important questions were addressed using recombinant DNA techniques (Fig. 4). First, do the short HIF-1α isoform and long HIF-1α isoform have similar DNA-binding activities? I.1 isoform has the same four amino acids upstream of the basic helix-loop-helix domain that is involved in DNA recognition and dimerization with ARNT (Fig. 4A), but the possibility existed that the absence of the 12 N-terminal amino acids could affect the HIF-1 functioning as a transcriptional factor. To determine whether short I.1 HIF-1α retains DNA binding activity similar to that of the I.2 isoform, we studied the binding of in vitro translated HIF-1α isoforms to HRE-containing oligodeoxyribonucleotide. Analysis of in vitro translated HIF-1α isoforms shows that electrophoretic mobility of both isoforms is in agreement with their estimated molecular mass (92.3 kDa for I.1 HIF-1α, 93.5 kDa for I.2 HIF-1α, and 86.6 kDa for ARNT) (Fig. 4B). Electrophoretic mobility shift assay data revealed that both short and long HIF-1α isoforms display the ability to bind HRE (Fig. 4C). These data confirm previous results of A. Gorlach et al. (40), showing that the lack of 12 amino acids adjacent to the basic helix-loop-helix domain does not abolish HIF-1 DNA binding activity. However, it has not been established yet whether these two isoforms display the same transcriptional activity. To compare the ability of I.1 and I.2 HIF-1α isoforms to activate the HRE-dependent transcription, HIF-1α and ARNT expression vectors were used to cotransfect cells with luciferase plasmid containing the hypoxia-
response element from the iNOS promoter. HRE-dependent luciferase expression was induced equally well by both isoforms, and they have similar transcriptional activity compared with human HIF-1α/H9251 (Fig. 4D). Therefore, both short and long isoforms retain the ability to bind to HRE and activate transcription of responsive HRE-containing genes. Finally, we examined whether the up-regulation of HIF-1α mRNA from untreated cells is assigned 1 relative unit for the presentation of results. Samples were normalized for β-actin mRNA expression. D, competitive RT-PCR for HIF-1α I.1 mRNA from T cells and 2B4 cells. Cells were stimulated as described above. The positions of the target and mimic RT-PCR products are indicated by the arrows. The same concentration of mimic was used for each sample. E, ConA-induced T cell activation. Upper panel, up-regulation of proinflammatory cytokines after intravenous injection of concanavalin A. C57BL/6 mice were treated with concanavalin A (20 mg/kg), and serum tumor necrosis factor-α and interferon-γ levels were determined by enzyme-linked immunosorbent assay. Tumor necrosis factor-α levels after 1.5 h and interferon-γ levels after 8 h were shown. Lower panel, quantitative RT-PCR for HIF-1α I.1 mRNA from ConA-activated T cells. T lymphocytes were in vivo stimulated by ConA as described under “Experimental Procedures.” 3-fold mimic dilutions are shown, where M1 represents maximal mimic concentration. The amount of HIF-1α mRNA from T cells derived from untreated mice is used as 1 relative unit. Samples were normalized for β-actin mRNA expression.

that transcription of VEGF and iNOS was enhanced in activated T cells (Fig. 5, A and B).

**DISCUSSION**

The main findings of this study are (i) the oxygen-independent up-regulation of HIF-1α mRNA in antigen receptor-activated T cells; (ii) dramatic differences in regulation of expression of I.1 and I.2 isoforms of HIF-1α mRNA; (iii) rapid and protein synthesis-independent up-regulation of I.1 HIF-1α mRNA, thereby identifying HIF-1α as an immediate early response gene; and (iv) similar transcriptional activity for both long and short HIF-1α protein isoforms, suggesting that they may be employed to target the same set of genes but at different physiological situations.

I.1 HIF-1α mRNA is not just expressed in a tissue-specific...
manner (39), but it is also expressed in a cell activation-dependent manner. This may provide an explanation for the increase in HIF-1α/H9251 mRNA in vivo (28–30) as cell activation during adaptation of live animals to hypoxia rather than to local tissue hypoxia. An activation-induced HIF-1α/H9251 mRNA expression described here explains the inconsistency between reports that described the hypoxia-independent HIF-1α mRNA expression in vitro (26, 27) and findings of HIF-1α mRNA up-regulation in vivo in animals maintained in hypoxic conditions (28–30). However, whether the difference in regulation of HIF-1α mRNA by hypoxia in vivo and in vitro could be explained by different mechanisms of oxygen sensing is yet to be established. The up-regulation of short HIF-1α isoform in vivo activated T cells during the course of proinflammatory cytokine-dependent fulminant hepatitis (Fig. 3E) strongly supports the physiological significance of these observations.

The findings described above indicate the functions of HIF-1α in T cell activities not related to hypoxia, which were not obvious from the original definition of hypoxia-inducible factor. These observations may reflect the previously unrecognized and important role of HIF-1α in both immune and "non-immune" functions of T cells. For example, it was shown that CD3-positive T cells and tumor-infiltrating lymphocytes express VEGF (55), thus implicating the potential role of T cells in neovascularization.

The observations presented in this work raise interesting questions as to why T cells activation leads to selective transcription of I.1 HIF-1α isoforms. It is also important to understand why activated T cells must employ an additional HIF-1α gene isoform, instead of stabilization of already synthesized HIF-1α protein, as was established in nonlymphoid cells in hypoxic conditions.

Why does HIF-1α exist in two isoforms? Two main possibilities could be considered. First, the 12-amino acid deficit in the N terminus of the shorter HIF-1α protein may result in changes in its interactions with, for example, cell cycle-regulating proteins such as p53 and therefore affect cell proliferation (23, 56).
This property of short HIF-1α could be beneficial for the T cell activation process. If this is true, then the short HIF-1α should have a different potential than the long isoform to interact with other proteins. Unfortunately, the functional differences between two HIF-1α isoforms derived from I.1 and I.2 mRNAs are yet to be discovered. Further studies should address the differences in DNA sequence specificity and transcriptional partners between these two HIF-1α isoforms and which process in activated T cells is regulated by short and/or long HIF-1α isoforms.

The selective up-regulation of I.1 HIF-1α isoform in activated T cells is best explained by differences in promoter regions of these two isoforms (Fig. 6A). The I.2 exon is located within the methylation-free Cpg island (39), which is commonly associated with the 5′-end of housekeeping genes (57), while the promoter of exon I.1 exhibits tissue-specific features (39). Moreover, the putative AP-1 binding site, which was shown to be an important regulator of gene expression in lymphocytes (58) is present in the exon I.1 promoter (39).

The alternative splicing of human HIF-1α mRNA was reported (59), but no alternative splicing in a starting exon has been discovered yet. It would be important to test whether human HIF-1α also consists of two or more differently regulated mRNA isoforms. Studies based on expression of these isoforms may shed light on mechanisms of HIF-1α action and dissociate functions of HIF-1 as transcription factor from its direct effects on other proteins such as p53 (23).

The oxygen-independent up-regulation of HIF-1α mRNA in antigen receptor-activated T cells (Fig. 1A) was an unexpected observation. This finding contradicted the firmly established mechanism that HIF-1α mRNA expression is oxygen tension-independent, while HIF-1α protein is degraded due pVHL-ubiquitin ligase activity (31, 32) in nonhypoxic conditions. This contradiction could not be resolved were it not for the alternatively spliced HIF-1α mRNA described by Wenger et al. (38). This allowed our group to study I.1 versus I.2 HIF-1α mRNA isoform expression patterns (Fig. 3). The TCR-mediated HIF-1α mRNA up-regulation can be determined and clarified as activation-dependent up-regulation of the short HIF-1α mRNA (Fig. 3C). This resolves the apparent contradiction, since the I.1 isoform behaves as was reported and expected and HIF-1α I.2 mRNA is constitutively expressed (Fig. 3D).

Rapid protein synthesis-independent up-regulation of I.1 mRNA (Fig. 2) identifies HIF-1α as an immediate early response gene family member (47). This suggests that HIF-1α is important very early after activation of lymphocytes by the antigen. The ability to discriminate between these possibilities depends on the knowledge of differences between long and short isoforms of HIF-1α. The gel shift assay and reporter gene transfection data (Fig. 4) suggest that both isoforms have similar transcriptional activity. It remains to be established whether these two isoforms have similar or different protein-protein interactions.

The oxygen tension-dependent, pVHL-ubiquitin-mediated HIF-1α degradation mechanism would be expected to operate with both short and long HIF-1α isoforms, because both isoforms contain an oxygen-dependent degradation domain. However, both isoforms would not appear to be equally well degraded at normoxic conditions, since the activation-induced expression of HIF-1α in T cells may be accompanied by a simultaneous decrease in the ability of pVHL to target the short HIF-1α for degradation. For example, it was shown that activation of G-protein-coupled receptor (38) results in HIF-1α induction, most likely by activity of GTPase Rac1 (60). This pathway may lead to stabilization of HIF-1α by the production of reactive oxygen species (61). It would be interesting to test in

**Fig. 5.** Transcription of HIF-1 target genes is enhanced in activated T cells. A, quantitative RT-PCR for VEGF mRNA from mouse Th1 cells. Cells were incubated with plate-bound anti-CD3 mAb and anti-CD28 mAb at 20% O2 at 37 °C for 24 h. The positions of the target and mimic RT-PCR products are indicated by arrows. Three-fold mimic dilution is shown, where M1 is maximal mimic concentration. The amount of VEGF mRNA from untreated cells is used as 1 relative unit. Samples were normalized for β-actin mRNA expression. B, quantitative RT-PCR for iNOS mRNA from mouse Th1 cells. Cells were activated as described above.
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Long (I.2) HIF-1α Expression

![Diagram of Long (I.2) HIF-1α Expression]

Short (I.1) HIF-1α Expression

![Diagram of Short (I.1) HIF-1α Expression]

FIG. 6. Model of TCR-mediated up-regulation of HIF-1α I.1 mRNA. Upper panel, constitutive expression of the long (I.2) HIF-1α isoform. Lower panel, T cell activation-dependent regulation of expression of the short (I.1) HIF-1α isoform.

Acknowledgments—We are grateful to Dr. R. Wenger and Dr. M. Gassmann for the gift of plasmids and helpful advice; Dr. G. Semenza and Dr. O. Hankinson for providing cDNAs of HIF-1α and HIF-1β; Dr. J. He-Li for the generous gift of Th1 and Th2 cells; Dr. M. Blagoslkinny for providing HRE-containing reporter plasmid; Dr. M. Kishiba for advice in developing RT-PCR procedures; and Dr. P. Smith and S. Starnes for the help in experiments and in preparing the manuscript.

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