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Role for casein kinase 2 in the regulation of HIF-1 activity

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Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor that plays a major role in cellular adaptation to hypoxia. The mechanisms regulating HIF-1 activity occurs at multiple levels *in vivo*. The HIF-1α subunit is highly sensible to oxygen and is rapidly degraded by the proteasome 26S in normoxia. Activation in hypoxia occurs through a multistep process including inhibition of HIF-1α degradation, but also increase in the transactivation activity of HIF-1. Several data indicate that phosphorylation could play a role in this regulation. In this report, we investigated the role of casein kinase 2 (CK2), an ubiquitous serine/threonine kinase, in the regulation of HIF-1 activity. Hypoxia was capable of increasing the expression of the β subunit of CK2, of inducing a relocalization of this subunit at the plasma membrane, of inducing nuclear translocation of the α subunit and of increasing CK2 activity. Three inhibitors of this kinase, DRB (5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole), TBB (4,5,6,7-tetrabromotriazole) and apigenin, as well as overexpression of a partial dominant negative mutant of CK2α, were shown to inhibit HIF-1 activity as measured by a reporter assay and through hypoxia-induced VEGF and aldolase expression. This does not occur at the stabilization process since they did not affect HIF-1α protein level. DNA-binding activity was also not inhibited. We conclude that CK2 is an important regulator of HIF-1 transcriptional activity but the mechanism of this regulation remains to be determined. Since HIF-1 plays a major role in tumor angiogenesis and since CK2 has been described to be overexpressed in tumor cells, this new pathway of regulation can be one more way for tumor cells to survive.

Key words: casein kinase 2; HIF-1; neoangiogenesis

HIF-1 (hypoxia-inducible factor-1) is a key regulator of cell response to reduced oxygen level. In response to hypoxic conditions, HIF-1 increases the expression of downstream target genes such as erythropoietin (EPO), vascular endothelial growth factor (VEGF) and glycolytic enzymes (aldolase A, enolase-α) and mediates adaptation of cells to decreased oxygen level.[1] The role of HIF-1 in the regulation of tumor growth by hypoxia via the initiation of angiogenesis is certainly the best example of such an adaptive response.[2]

Oncogene activation and tumor-suppressor inactivation result in deregulated cellular proliferation. However, most tumors grown larger than 1 mm^3 contain regions of low oxygen tension (hypoxia) due to an imbalance between oxygen supply and consumption. Formation of new blood vessels or "neoangiogenesis" is thus essential for further tumor growth. It is also important to allow tumor cell dissemination at distant sites, *i.e.*, metastasis.

Several studies using HIF-1 mutant cells have shown that HIF-1 has a profound effect on tumor biology. For example, tumors grown from HIF-1α-defective embryonic stem cells display abnormal vascularity and reduced growth rate.[3] Moreover, HIF-1 is upregulated in a broad range of cancers and there is a significant correlation between tumor grade, vascularization and HIF-1α overexpression.[4,5] This pattern of expression suggests that the tumor cells are responding to hypoxia by HIF-1-mediated expression of angiogenic proteins. Among them, VEGF is probably the most potent one and its expression is regulated by HIF-1. In addition to promoting VEGF release, HIF-1 is also essential for tumor cell adaptation to hypoxia, notably via increasing glycolysis capacity.[6]

HIF-1 is a heterodimeric transcription factor composed of 2 members of the basic helix-loop-helix (bHLH) proteins containing Per-ARNT-Sim (PAS) motif, HIF-1α and ARNT (aryl receptor nuclear translocator).[7] HIF-1α is the subunit regulated by hypoxia and the oxygen-dependent regulation occurs at multiple levels *in vivo*.[8]

Under normoxic conditions, HIF-1α subunit, although constitutively expressed, is rapidly degraded by the ubiquitin-proteasome pathway such that almost no HIF-1α protein accumulates.[9] HIF-1α is targeted by an ubiquitin-ligase complex containing pVHL protein (Von Hippel-Lindau protein)[10–12] that recognizes 2 hydroxylated proline residue (P564 and P402) in the oxygen-dependent degradation (ODD) domain of HIF-1α. This process is regulated by an O_2-sensitive enzyme called HIF-1α prolyl-hydroxylase (HIF-PH).[13,14]

In hypoxic cells, the degradation pathway is inhibited and HIF-1α accumulates and migrates into the nucleus allowing the dimerization with the nuclear ARNT subunit. The active heterodimeric complex recognizes hypoxia-responsive element (HRE) present in the promoter of the hypoxia-responsive genes.

In addition to the regulation of the HIF-1α protein level, HIF-1 activity is also regulated by oxygen. This mainly occurs through the hydroxylation of an asparagine (N803) by an oxygen-sensitive asparagine hydroxylase.[15] Hydroxylation of this residue prevents the interaction of HIF-1α with the coactivator CBP/p300 and hence HIF-1 activity in normoxia.[16] In hypoxia, this modification no longer occurs and HIF-1 transcriptional activity is switched on. In addition, phosphorylation events also play a role as demonstrated by several studies: the activity of the MAP kinases ERK1/2 is needed for the transcriptional activity of HIF-1 but not for the stabilization of HIF-1α.[17,18]

However, the C-terminal transactivation domain (TAD-C) of HIF-1α is not directly phosphorylated by ERKs[19] but a negative charge on threonine 796, located in the C-terminal transactivation domain of HIF-1α, seems to be essential for the interaction with CBP/p300 and hence for the activity of HIF-1.[20] These results suggest that kinases other than ERK1/2 could be involved. The minimal consensus CK2 phosphorylation site is S/T-X-X-E/D and acidic amino acids around are favorable.[21] Computer analysis indicated that S551, S581, T700, T796 and S786 are present in putative CK2 phosphorylation sites.

Casein kinase 2 is a ubiquitous serine/threonine protein kinase found in eukaryotic cells. CK2 is involved in a variety of cellular processes including metabolism, signal transduction and transcription. CK2 has also been described to be overexpressed in cancer cells.[22] The human CK2 is composed of 2 catalytic subunits, α and α′, and 2 regulatory β subunits with molecular masses of 43, 38 and 28 kDa, respectively. The heterotetramer holoenzyme exists as $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$, or $\alpha'_2\beta_2$ tetramers.[23] A 1:1 stochiometry of the α and β subunits is required for optimal CK2 activity. The α catalytic subunit is stimulated by the β regulatory subunit, which undergoes autophosphorylation.[24] This kinase is present in both the cytoplasmic and nuclear compartments. CK2 is known to phosphorylate a variety of transcription factors *in vivo* and *in vitro* so that their activities are either positively or negatively

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modulated. The phosphorylation by CK2 can affect transcription factors by changing the DNA-binding activity as it is observed for c-Jun[25] and Sp1,[26] by modulating their transcriptional activities such as for MyoD,[27] HIV-1,[22] or IRF-1,[28] and by affecting protein stability, as observed for IκBα,[29,30] PTEN[31] and connexin 45.6.[32]

In this work, by using classical selective inhibitors of this kinase such as apigenin,[33–36] DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole)[22,37,38] and TBB (4,5,6,7-tetrabromobenzotriazole),[39–41] we investigated the effect of hypoxia on CK2 activity and the role that this kinase may play to regulate HIF-1 activity.

Material and methods

Cell culture

Hepatoma cells were grown in DMEM (Gibco, Gaithersburg, MD) containing 10% fetal calf serum, 100 U/ml penicillin G, 100 μg/ml streptomycin and 50 ng/ml amphotericin B. COS-7 cells were maintained in DMEM (Gibco) containing 10% fetal calf serum, 100 U/ml penicillin G, 100 μg/ml streptomycin and 50 ng/ml amphotericin B as well as 20 mM HEPES. Normoxia (21% O_2) and hypoxia (1% O_2) incubations were performed in serum-free CO_2-independent medium (Gibco) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, 50 ng/ml amphotericin B (Gibco) and 10 mM glutamine. For the incubation, DRB (BioMol, Plymouth Meeting, PA), apigenin (Sigma, St. Louis, MO), 4,5,6,7-tetrabromobenzotriazole (Calbiochem, San Diego, CA) and/or 3,4-dihydroxybenzoate (Sigma) were added to the CO_2-independent medium.

Western blot analysis

Total cell extracts were prepared from HepG2 grown in T25 cm^2 flasks at 90%. After the incubations, cells were washed with ice-cold PBS and lysed using lysis buffer [Tris 20 mM, pH 7.5 (Merck, Darmstadt, Germany), KCl 150 mM (Merck), EDTA 1 mM, Triton X-100 1% (Janssen Chemica, Beerse, Belgium), protease (Complete; Boehringer/Roche, Mannheim, Germany) and phosphatase (25 mM Na_2VO_4, 10 mM PNPP, 10 mM β-glycerophosphate and 5 mM NaF) inhibitors]. The lysate was centrifuged 5 min at 14,000*g* at 4°C and the supernatant was kept frozen. Extracts were separated on a 10% or 12% SDS-PAGE and then transferred to a PVDF membrane (Pharmacia, Kalamazoo, MI). After blocking in TBS containing 0.1% Tween and 5% dried milk, the blot was probed with anti-HIF-1α antibodies [Transduction Laboratories, Clontech [Mountain View, CA] dil 1/1,000; secondary antibody: antimouse (Amersham) dil 1/2,000], with CK2α [SC-9030 (Santa Cruz Biotechnology, Santa Cruz, CA), dil 1/1,000; secondary antibody: antirabbit (Amersham), dil 1/2,000] or CK2β antibodies [Calbiochem, dil 1/1,000; secondary antibody: antimouse (Amersham), dil 1/2,000]. Anti-α-tubulin antibodies [Innogenex, San Ramon, CA dil 1/2,000; secondary antibody: antimouse (Amersham), dil 1/2,000] was used to probe α-tubulin as a control for total amount of proteins loaded on the gel. Chemiluminescent detection was performed using horseradish peroxidase conjugated to the secondary antibody.

CK2 in vitro *kinase assay*

Cells were collected by cell scraping into a lysis buffer [Tris 10 mM, pH 7.5 (Merck), NaCl 150 mM (Merck), EDTA 1 mM, EGTA 1 mM, Triton X-100 1% (Janssen Chemica), protease inhibitor (Complete; Boehringer/Roche) and phosphatase inhibitor cocktail]. Whole cell homogenates were centrifuged at 12,500*g* for 15 min at 4°C and the supernatants were transferred to fresh microcentrifuge tubes; 2.5 μg of anti-CK2α antibodies (Santa Cruz) were then added and incubated for 1 hr at 4°C and CK2 was immunoprecipitated by adding 60 μl of protein A/protein G-coated sepharose beads (Oncogene, Cambridge, MA). The immunoprecipitated samples were then incubated with 200 μM substrate peptide in 40 μl Assay dilution buffer I (casein kinase 2 assay kit; Upstate, Charlottesville, VA) and 10 μCi [γ-^{32}P]-ATP (NEN) for 20 min at 30°C. The reaction was then stopped by adding 20 μl of TCA 40%. The sample was centrifuged for 1 min at 12,500*g* and the supernatant was loaded on phosphocellulose membrane Spinzyme columns (Pierce, Rockford, IL). The columns were rinsed twice with 75 mM phosphoric acid. The radioactivity associated with the columns was then measured. Results are presented in cpm.

Transfection experiments and reporter gene assay

To assay for the transcriptional activity of HIF-1, the pGL3-SV40/6HRE reporter vector containing an artificial promoter with TATA box and 6 copies of the EPO HRE cis-element upstream of the firefly luciferase gene was used. pGL3-SV40 was used as the negative control. HepG2 transfection was performed in a 24-well plate with Superfect transfection reagent (Qiagen, Chatsworth, CA); 200 ng of PGL3-SV40/6HRE was cotransfected with 100 ng of the control vector pRL-SV40 (Promega, Madison, WI) and with 1 μg expression or null vector. Vectors expressing wild-type CK2α or a kinase dead CK2α acting as a partial negative dominant mutant (K68A) were used and pCMV-Myc (pMyc) was used as null vector. Twenty-four hours posttransfection, the medium was replaced by CO_2-independent medium (Gibco) without serum and with or without CK2 inhibitor and cells were incubated for 16 hr in either 20% or 1% O_2. After the incubation, the luciferase activity was measured. Luciferase activity was quantified in a luminometer using the Dual-Luciferase-Reporter System (Promega). Experiments were performed in triplicates. Results are expressed as means of the ratio between firefly luciferase activity and renilla luciferase activity. To analyze the effect of CK2 inhibitors on HIF-1α TAD-N and TAD-C, pGal/α530-826, pGal/α775-826, pGal/α530-611/ARNT-ta, pGAL/ARNT-ta and pSG424 were used with pUAS-TK Luc as the rapporter plasmid.[42]

Immunofluorescence

HepG2 cells grown on glass coverslip were incubated in normoxia or hypoxia with or without inhibitor. After the incubation, the medium was removed and cells were fixed 10 min with PBS containing 4% paraformaldehyde (Merck; HIF-1α, CK2β). The fixed cells were washed 3 times with PBS and permeabilized with a solution of PBS-Triton X-100 1% (Sigma). For immunolocalization of CK2α, cells were fixed and permeabilized with methanol 80%-acetone 20% at −20°C for 10 min. After 3 washing steps with PBS + BSA 3% (Sigma), cells were incubated at 4°C for 2 hr with the anti-HIF-1α antibody (dil 1/100; Transduction Laboratories), with the anti-CK2β antibody (dil 1/100; Calbiochem) or with the anti-CK2α antibody (dil 1/100; SC-6497; Santa Cruz). Then, cells were washed 3 times with PBS + BSA 3% and the secondary antibodies conjugated to Alexa fluorochrome (488; dil 1/500; Molecular Probes, Eugene, OR) were added for 1 hr. Afterward, the cells were washed 3 times with PBS + BSA 3%. For nucleus labeling, the cells were incubated with TOPRO-3 (dil 1/80; Molecular Probes). Finally, the cells were mounted in mowiol. Observations were performed using a confocal microscope (Leica, Vienna, Austria). Semiquantitative observations were performed with a constant photomultiplier (PMT) value.

Colorimetric assay for HIF-1

HIF-1 DNA-binding activity was measured using a colorimetric assay developed in our laboratory[43] and sold by Active Motif (Trans-AM; Active Moti, San Diego, CA). The assay was performed as recommended by the supplier. For CBP detection, nuclear extracts were prepared with 0.3 M NaCl instead of 0.8 M in order to maintain protein-protein interaction. Detection was performed using anti-CBP antibody (SC-1211; Santa Cruz; dilution 500×).

VEGF assay

VEGF secreted in the incubation medium was assayed by an ELISA (Quantikine; R&D Systems, Minneapolis, MN) according to the procedure provided by the supplier. Results are expressed in ng of VEGF reported to μg of proteins assayed by the Folin method.

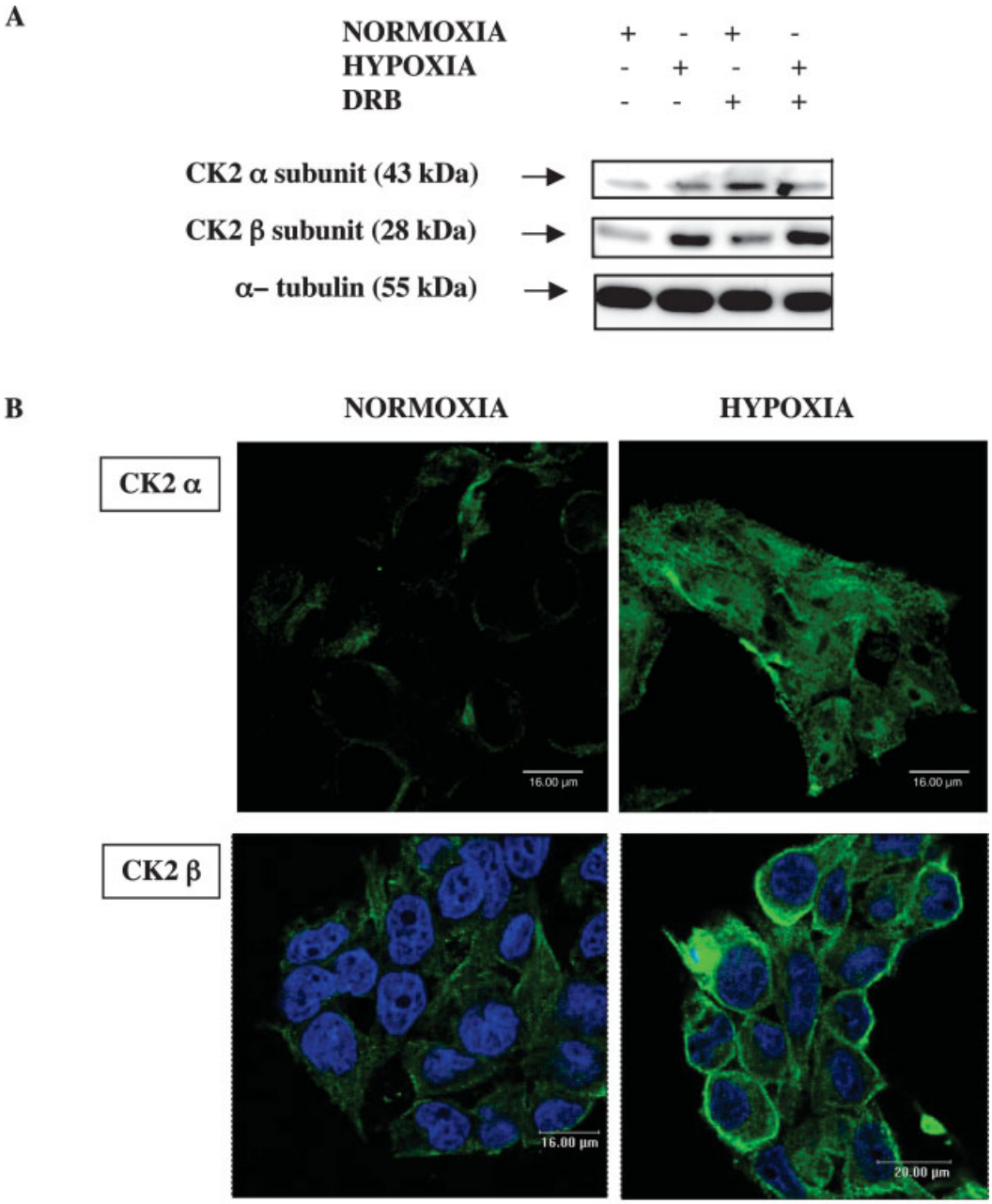
A
NORMOXIA + - + -
HYPOXIA - + - +
DRB - - + +
CK2 α subunit (43 kDa)
CK2 β subunit (28 kDa)
α– tubulin (55 kDa)
B
NORMOXIA
HYPOXIA
CK2 α
CK2 β
16.00 µm
16.00 µm
16.00 µm
20.00 µm
C
CK2 activity (cpm)
50000
40000
30000
20000
10000
0
normoxia
N + DRB
hypoxia
H + DRB

FIGURE 1.

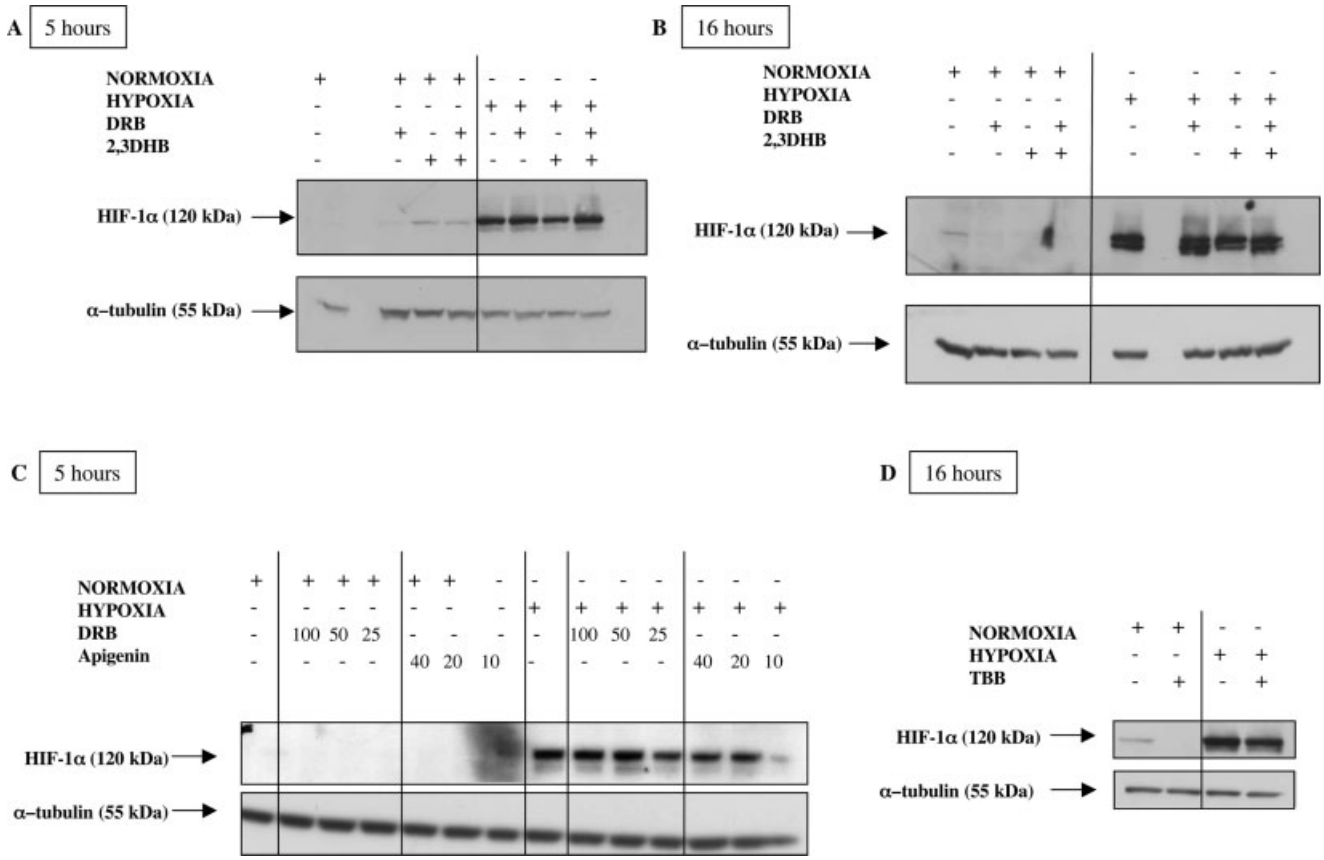

FIGURE 2 – Effect of DRB on HIF-1α protein level. HepG2 cells were incubated 5 (*a*, *c*) or 16 (*b, d*) hr in either normoxia or hypoxia in the presence or in the absence of DRB at 50 μM and or 3,4DHB at 100 μM (*a, b*) of different concentrations of DRB or apigenin (μM *c*) or 10 μM TBB (*d*). After the incubation, the cells were lysed and total protein extracts were resolved by SDS-PAGE. HIF-1α was then revealed by Western analysis. A Western blot for α-tubulin was performed to check for the total amount of proteins loaded on the gel.

Real-time RT-PCR

After 16 hr of incubation in normoxia or in hypoxia, total RNA was extracted with the RNAgents total RNA isolation kit (Promega) according to the instructions of the manufacturer. mRNAs were then retrotranscribed using oligodT. Aldolase mRNA expression level was quantified by real-time PCR using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) according to the instructions of the manufacturer. Values were then normalized to the relative amounts of the housekeeping gene α-tubulin. Each gene was amplified using the appropriate specific primers.

Results

Effect of hypoxia on CK2 α and β protein level

Several studies indicate that CK2 is required for cell viability. Moreover, a heat shock stress induces a relocalization of protein kinase CK2 in the nucleus, increasing the activity of the kinase in this compartment but the exact mechanism underlying the regulation of this heat-induced response of CK2 is not known.[44] The possible relocalization of this kinase in hypoxic conditions was examined and the protein level of both subunits was quantified. Western blot analysis (Fig. 1*a*) and immunofluorescence studies (Fig. 1*b*) were performed. HepG2 cells were incubated in the absence of serum in either hypoxia (1% O_2) or normoxia (21% O_2) for 16 hr. A slight increase in the amount of CK2α present in whole cell extracts was observed when cells were exposed to hypoxia. Moreover, hypoxia markedly increased the expression of the β regulatory subunit, as evidenced by Western blot and immunofluorescence studies. Immunolocalization of CK2α showed that while it was mainly localized in the cytosol in normoxia, it seemed to translocate into the nucleus in hypoxia. On the other hand, CK2β was localized at the plasma membrane in hypoxia while it was more cytoplasmic in normoxia. These results indicate that CK2 subunits can exist in different cellular compartments, where they might exert distinct functions, and that this process can be regulated by hypoxia. The actual CK2 activity was then measured in whole cell extract. Hypoxia increased by 5-fold the activity of this kinase, which can be inhibited by DRB (Fig. 1*c*).

FIGURE 1 – Effect of hypoxia on casein kinase 2 protein level and activity. HepG2 cells were incubated 16 hr in either normoxia or hypoxia in the presence or the absence of 50 μM DRB. (*a*) After the incubation, the cells lysed and total protein extracts resolved by SDS-PAGE. CK2α and CK2β were then revealed by Western analysis. A western blot for α-tubulin was performed to check for the total amount of proteins loaded on the gel. (*b*) After the incubation, the cells were fixed and immunolabeled for CK2α and CK2β. Nuclei were stained with TOPRO-3 (blue). Observation was performed in semiquantitative confocal microscopy. (*c*) After the incubation, CK2α was immunoprecipitated and in vitro CK2 activity was determined. Results are expressed in cpm.

Effect of CK2 inhibition on HIF-1α stability

The mechanism of the activation of HIF-1 by hypoxia occurs at different levels involving stabilization of the HIF-1α subunit, regulation of the DNA binding and activation of the transcriptional activity. Phosphorylations seem to be involved for the stabilization of the HIF-1α subunit in hypoxia. Indeed, serine/threonine kinase inhibitors such as 2-aminopurine are able to

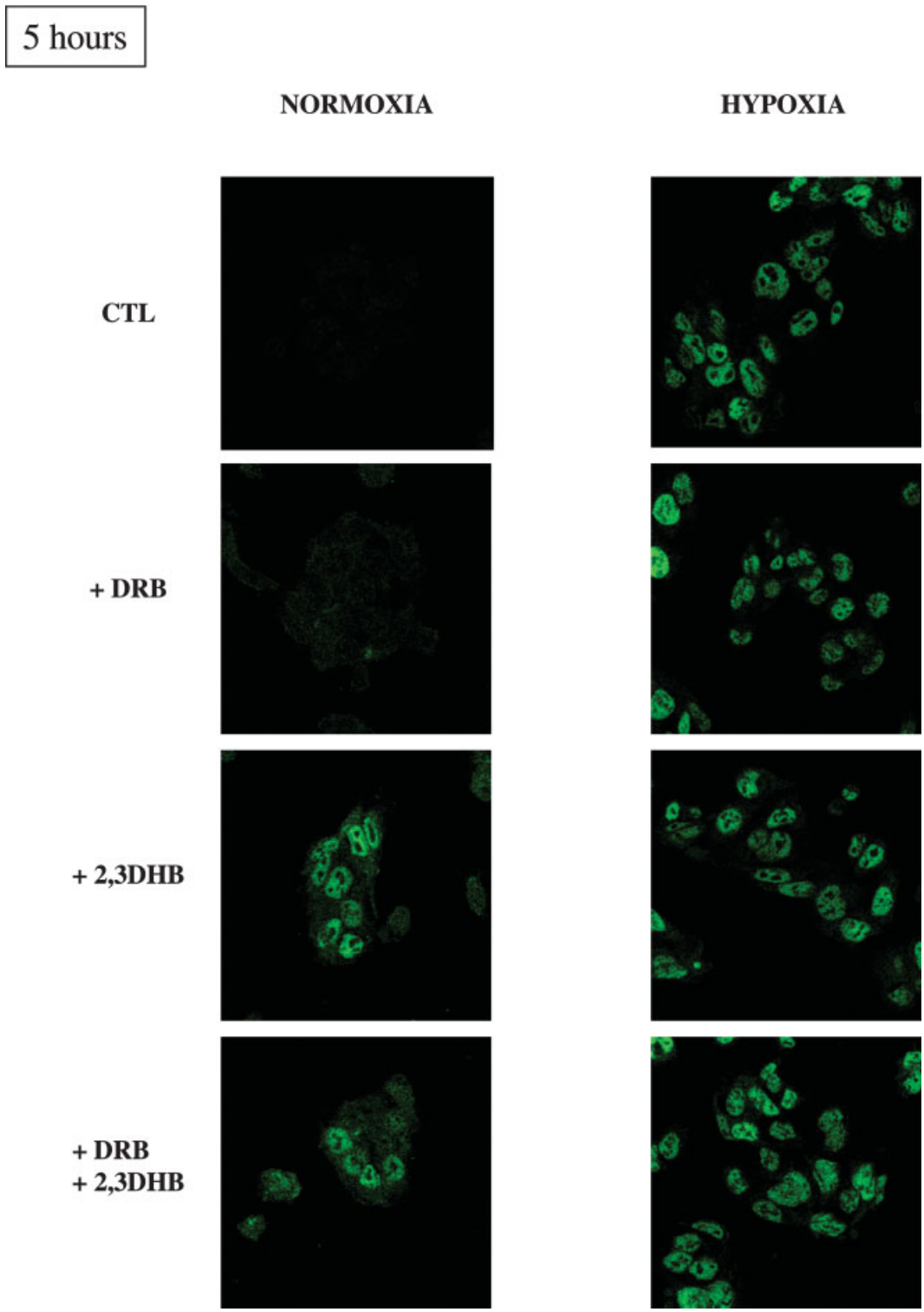

FIGURE 3 – Effect of DRB on HIF-1α protein level. HepG2 cells were incubated 5 hr in either normoxia or hypoxia in the presence or in the absence of DRB at 50 μM and with or without 3,4DHB at 100 μM. After the incubation, the cells were fixed and immunolabeled for HIF-1α. Observation was performed in semiquantitative confocal microscopy.

impair the hypoxia-induced HIF-1α stabilization.[45] Moreover, several reports have shown that phosphorylation may affect intrinsic protein stability as it was described for the IκB inhibitor protein.[29,30,46] Indeed, stimuli such as TNF-α cause rapid phosphorylation of the C-terminal PEST domain of IκB leading to ubiquitinylation and degradation of the inhibitor subunit by the proteasome 26S. NF-κB protein then translocates to the nucleus and activates gene expression. Some of the phosphorylations controlling IκB degradation are performed by casein kinase 2.[38] The similarities between NF-κB and HIF-1α concerning

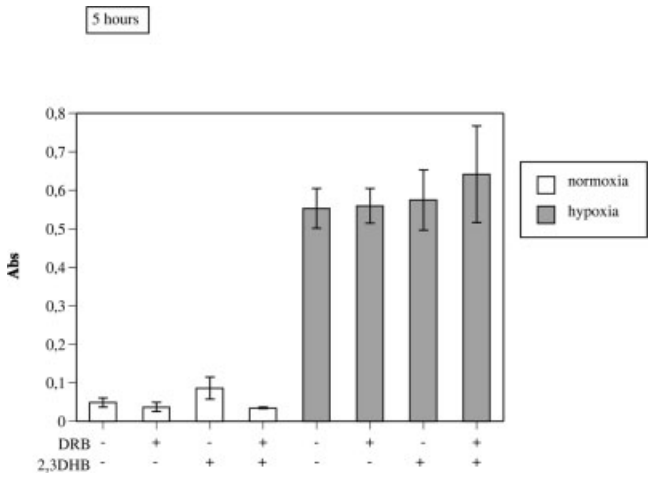

FIGURE 4 – Effect of DRB on HIF-1 DNA-binding activity. COS-7 cells were incubated 5 hr in either normoxia or hypoxia in the presence or in the absence of DRB at 50 μM and with or without 3,4DHB at 100 μM. After the incubation, the cells were lysed and nuclear protein extracts were tested for DNA binding to a probe containing the HRE sequence. The presence of the DNA-bound transcription factor is then detected by anti-HIF-1α antibodies and revealed by colorimetry. Results are presented as mean ± SD for triplicates.

sequestration in the cytoplasm, degradation by the proteasome 26S in normoxia and translocation into nucleus under activating conditions were very interesting and led us to study the influence of CK2 inhibition on the hypoxia-induced stabilization of HIF-1α but also on the stabilization induced by inhibition of the HIF-prolyl hydroxylase. 3,4-dihydroxybenzoate (3,4DHB) was used to inhibit this enzyme.[47] Cells were incubated 5 or 16 hr in the presence or in the absence of DRB,[22,38,48] a specific inhibitor of CK2α, with or without 3,4DHB, and Western blotting analyses were performed (Fig. 2*a* and *b*). HIF-1α is continuously degraded in normoxia and accumulates in hypoxia. 3,4DHB inhibited the normoxia-induced degradation of HIF-1α, which then accumulates after 5-hr incubation, but to a lower extent than in hypoxia. DRB did not affect HIF-1α protein level either in normoxia, in hypoxia, or in the presence of 3,4DHB. However, it is interesting to note that DRB modifies the electrophoresis pattern of HIF-1α, as a third band appeared on the gel, with an apparently lower molecular weight. Similar results were obtained in the immunofluorescence studies (Fig. 3).

Different concentrations of DRB and of apigenin, another CK2 inhibitor, were also tested in Western blot without any effect of DRB and a slight decrease in HIF-1α induced by apigenin (Fig. 2*c*). TBB, a more specific inhibitor of CK2, was also tested: a slight decrease in the amount of HIF-1α was observed in the presence of this inhibitor (Fig. 2*d*). These different results suggest that CK2 is probably not much involved in regulating HIF-1α synthesis and degradation in normoxia or in hypoxia.

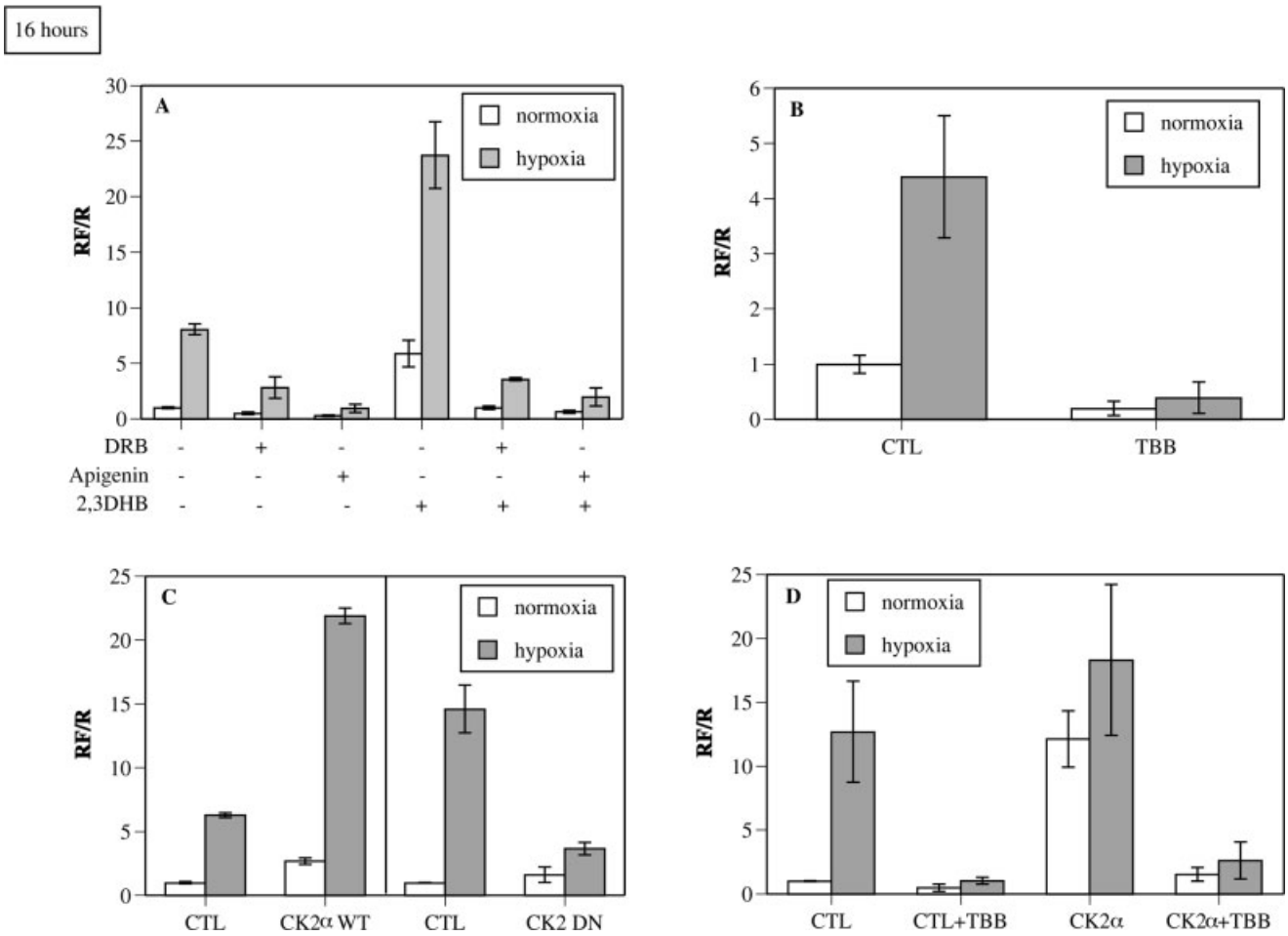

FIGURE 5 – Effect of DRB, apigenin (*a*), TBB (*b*, *d*) and CK2α wild-type or dominant negative mutant overexpression (*c*, *d*) on HIF-1 transcriptional activity. HepG2 cells were cotransfected with pGL3-SV40-6HRE together with the pRL-SV40 and with an expressing vector for CK2α or the null pMyc vector (CTL). The cells were incubated 16 hr in either normoxia or hypoxia in the presence or in the absence of apigenin at 40 μM or DRB at 50 μM, TBB at 10 μM and with or without 3, 4DHB at 100 μM. After the incubation, the cells were lysed for luciferase assay. Data represent the ratio between firefly luciferase activity and renilla luciferase activity (RF/R). Results are presented as mean ± SD for triplicates.

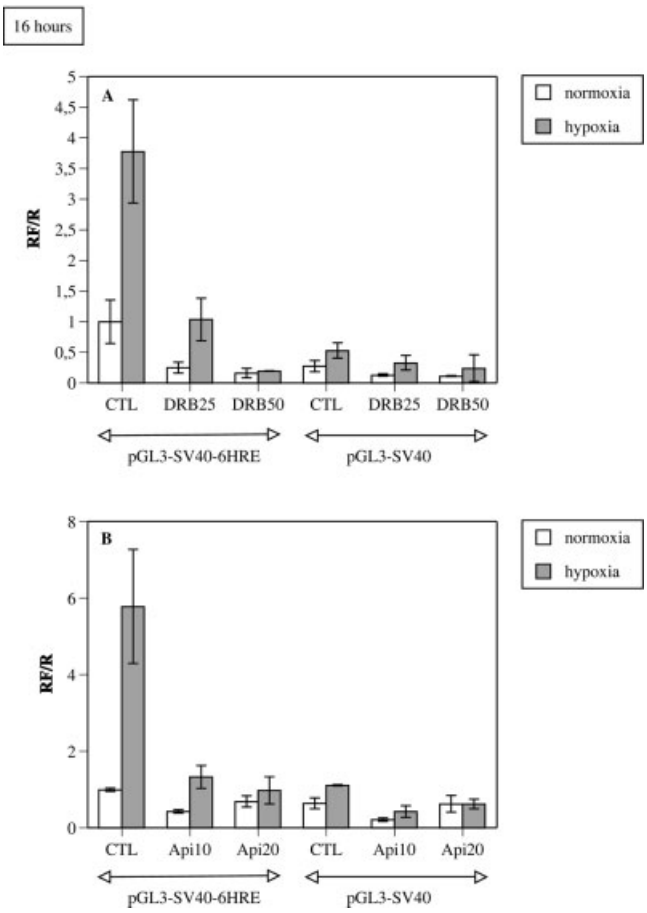

FIGURE 6 – Effect of DRB and apigenin on HIF-1 transcriptional activity. HepG2 cells were cotransfected with pGL3-SV40-6HRE or pGL3-SV40 together with the pRL-SV40 and the null pMyc vector. The cells were incubated 16 hr in either normoxia or hypoxia in the presence or in the absence of different concentrations of DRB (*a*) or apigenin (*b*; μM). After the incubation, the cells were lysed for luciferase assay. Data represent the ratio between firefly luciferase activity and renilla luciferase activity (RF/R). Results are presented as mean ± SD for triplicates.

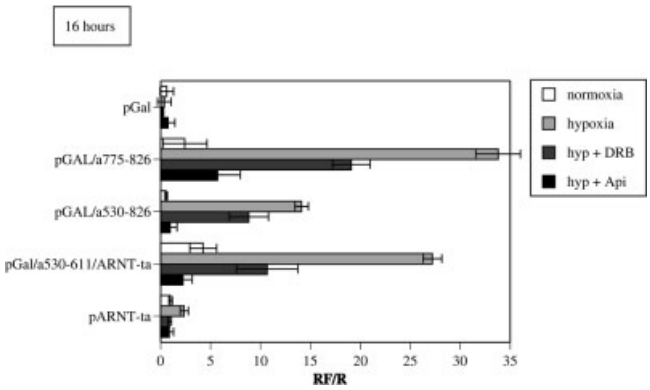

FIGURE 7 – Effect of DRB and apigenin on HIF-1α TAD-N and TAD-C activity. HepG2 cells were cotransfected with pUAS-TK Luc together with the pRL-SV40 and with pGal/α530–826, pGal/α775–826, pGal/α530–611/ARNT-ta, pGAL/ARNT-ta, or pSG424 as the negative control. The cells were incubated 16 hr in either normoxia or hypoxia in the presence or in the absence of DRB at 50 μM or apigenin at 20 μM. After the incubation, the cells were lysed for luciferase assay. Data represent the ratio between firefly luciferase activity and renilla luciferase activity (RF/R). Results are presented as mean ± SD for triplicates.

Effect of CK2 inhibition on HIF-1 transcriptional activity

The second step of HIF-1 activation is DNA binding. The DNA-binding capacity of HIF-1 was tested using a DNA-binding assay based on the use of multiwell plates coated with a cold oligonucleotide containing the consensus binding site of HIF-1. The presence of the DNA-bound transcription factor is then detected by HIF-1α antibodies and revealed by colorimetry. The specificity of this assay was demonstrated in a competition experiment using the wild-type or a mutated probe.[49] Using this technique, we showed that DRB did not directly influence HIF-1 DNA-binding capacity in hypoxia (Fig. 4).

Finally, regulation of the transcriptional activity directly through modification of HIF-1α or of the coactivators may also play a role. As shown in Figure 5(*a*), DRB was able to decrease by 2- to 3-fold the luciferase activity of the 6HRE reporter system in hypoxia. Apigenin and TBB were also tested and had the same effect on the luciferase reporter gene expression (Fig. 5*a* and *b*). Similarly, both inhibitors could markedly decrease HIF-1 transcriptional activity in hypoxia in the presence of 3,4DHB. The higher HIF-1 transcriptional activity observed in the presence of this molecule was probably due to its inhibitory effect of the asparagine hydroxylase. We verified the specificity of DRB and apigenin by comparing their effect on pGL3-SV40/6HRE with their effect using pGL3-SV40, which does not contain HIF-1 DNA-binding site. A concentration-dependent inhibition of luciferase activity induced by DRB was observed when 6HRE is driving luciferase expression while no effect was evidenced when using pGL3-SV40 (Fig. 6*a*). Similar results were obtained using apigenin (Fig. 6*b*). We verified that the effect was specific for CK2 inhibition by overexpressing the α subunit of this kinase. The overexpression of CK2α increased HIF-1 activity by 6-fold in normoxia and in hypoxia (Fig. 5*c*); again, this effect was completely prevented by its inhibition using TBB (Fig. 5*d*). Finally, a kinase dead CK2α acting as a partial negative dominant mutant (K68A) was overexpressed. The results show that this mutant markedly inhibited HIF-1 activity (Fig. 5*c*). These results suggest that active CK2 is needed for HIF-1 transcriptional activity.

In order to investigate the influence of CK2 inhibitors on intrinsic transactivating activity of each transactivating domain of HIF-1α (TAD-N and TAD-C), plasmids expressing fusion proteins composed of HIF-1α transactivating domains linked to a heterologous DNA-binding domain (Gal4 DBD) were used as described by Pugh *et al*.[42] The negative control containing the ARNT transactivating domain showed little response to hypoxia or to CK2 inhibitors while the positive control (aa 530–826 of HIF-1α) displayed a large increase in activity under hypoxia compared to normoxia. This increase was partly inhibited by DRB and totally blocked in the presence of apigenin (Fig. 7). TAD-N (aa 530–611) as well as TAD-C (775–826) showed similar properties, indicating that CK2 activity is needed for the activity of both transactivating domains of HIF-1α in hypoxia.

A common mechanism by which TAD-N and TAD-C exert their transactivating activity is by recruiting coactivators, CBP being one of them. In order to investigate whether CK2 inhibitors would interfere with HIF-1α-CBP interaction, CBP bound to HIF-1 when HIF-1 is trapped onto a DNA probe containing one HRE and fixed into a well was detected by a specific antibody. If more CBP was detected in hypoxia than in normoxia, probably because more HIF-1 is present and binds to the HRE probe, no effect of DRB was observed in hypoxia (Fig. 8). These results suggest that CK2 inhibition does not affect CBP-HIF-1 interaction in hypoxia.

Influence of CK2 inhibition on hypoxia-induced VEGF expression

VEGF is one the most responsive genes to hypoxia,[50–52] whose expression is driven by HIF-1 but also by AP-1 in hypoxia.[53,54] The effects of DRB on the basal VEGF synthesis and on the hypo-

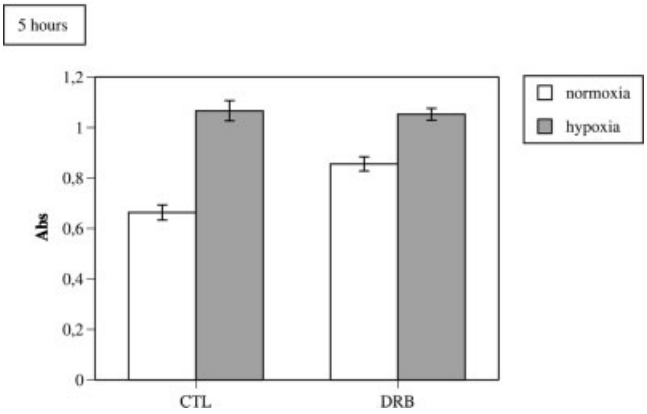

FIGURE 8 – Effect of DRB on CBP-HIF-1 interaction. COS-7 cells were incubated 5 hr in either normoxia or hypoxia in the presence or in the absence of DRB at 50 μM. After the incubation, the cells were lysed and nuclear protein extracts was tested for DNA binding to a probe containing the HRE sequence. The presence of CBP bound to HIF-1 trapped onto the HRE probe is then detected by anti-CBP antibodies and revealed by colorimetry. Results are presented as mean ± SD for triplicates.

xia-induced VEGF secretion were tested in order to study the physiologic relevance of the CK2 inhibition in HIF-1 activation. This inhibitor had no effect on the basal secretion of VEGF in normoxia. On the other hand, DRB decreased the hypoxia-induced increase in VEGF secretion (Fig. 9*a*).

Aldolase is also a well-known HIF-1 target gene. The mRNA level of this gene was followed by real-time RT-PCR. Hypoxia increased by 7-fold the quantity of aldolase mRNA. This increase was inhibited by 50% in the presence of DRB at 50 μM and nearly totally in the presence of apigenin at 20 μM. DRB and apigenin did not decrease aldolase mRNA in normoxia (Fig. 9*b*).

These results are similar to the ones observed in hypoxia when measuring the transcriptional activity of HIF-1 using an HRE-driven reporter assay as well as when assessing its transactivating domain activity.

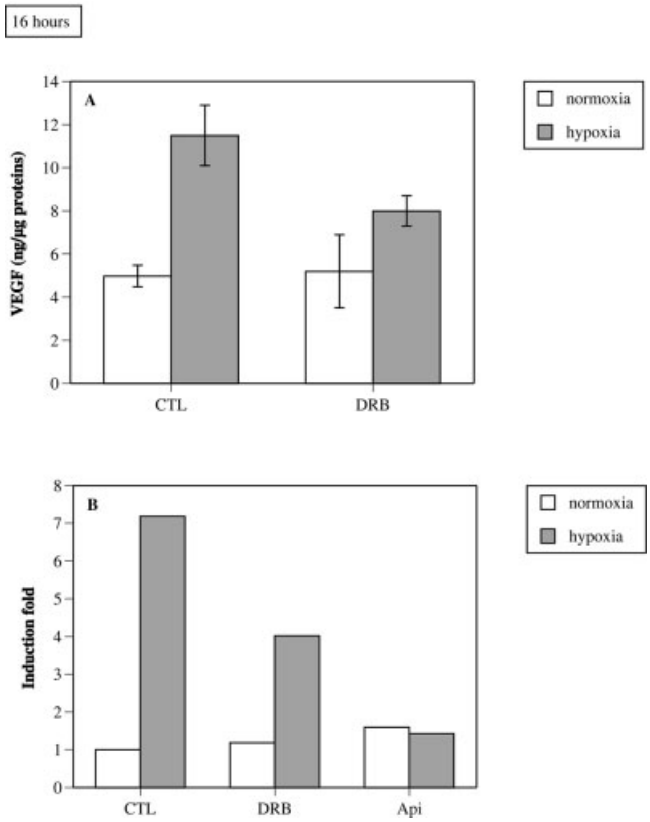

FIGURE 9 – Effect of DRB on VEGF secretion and aldolase mRNA expression. HepG2 cells were incubated 16 hr in either normoxia or hypoxia in the presence or in the absence of DRB at 50 μM or apigenin at 20 μM. (*a*) After the incubation, the medium was recovered for VEGF assay by ELISA and the cells were lysed for protein assay. Results are expressed in ng VEGF/μg proteins and presented as mean ± SD for triplicates. (*b*) After incubation, total RNA was extracted and retrotranscribed into cDNA. A real-time PCR has been performed with specific primers for aldolase as well as with primers specific for α-tubulin, a housekeeping gene. Results are expressed in induction level by comparison with the reference condition, normoxia.

Discussion

In challenging conditions such as heat shock, oxidative stress and decreased oxygen levels, cells react by inducing physiologic stress responses enabling them to survive. This involves activation of signaling pathways, activation of transcription factors, posttranscriptional modifications of proteins; hence, many proteins are playing a role in the adaptive response to the initial stress.

To activate the transcription of the O_2-dependent genes, HIF-1 transcription factor is regulated through different mechanisms involving stabilization of the HIF-1α subunit, regulation of the DNA-binding capacity and modulation of the transactivating activity. Most commonly, posttranslational regulation is achieved by interactions with different proteins or by phosphorylations. The main regulatory event involved in switching on the transcriptional activity of HIF-1 is the inhibition of HIF-1α hydroxylation on N803 in hypoxia.[15] However, phosphorylation also seems to play a role as it was shown that ERK1/2 MAPK are important to increase HIF-1 activity in hypoxia in some cell types.[17,18] Since HIF-1 activation by hypoxia occurs in all cell types while ERK1/2 is not important for this activation in all of them, other phosphorylation events may take place. In this work, we demonstrated that CK2 could be important for regulating HIF-1 activity in hypoxia.

In order for a kinase to play a role in the hypoxia-induced activation of HIF-1, its activity should be modulated in these conditions. Indeed, results shown in Figure 1 indicate that hypoxia increased CK2 activity by 5-fold. The regulation of CK2 activity is not very well known: change in ratio of α to β subunits as well as in subcellular localization may play a role.[55] Hypoxia did indeed increase the expression of the β subunit and induced a relocalization of this subunit to the plasma membrane. In addition, CK2α translocation into the nucleus was observed. This is an interesting observation since CK2 could hence phosphorylate different substrates under hypoxia, such as transcription factors and/or coactivators, thus modulating their activity. This could be the case for HIF-1. However, the consequence of these events on actual CK2 activity is still unknown. The β subunit has a cyclin-like destruction box in its amino terminus,[56] but the question of whether it targets the protein for proteolysis is still unanswered. It is possible that the hypoxia-induced increase in CK2β protein level observed in this work results from an inhibition of this putative degradation process.

The data presented here indicate that the activity of CK2 is required for HIF-1 activity. Indeed, its inhibition led to a decreased luciferase expression in a reporter system but also to a decrease in the hypoxia-induced VEGF and aldolase overexpression. This regulation occurs at the transactivation level since neither the HIF-1α protein level nor the DNA-binding capacity of HIF-1 was altered by these inhibitors. In addition, DRB and apigenin affected transactivation activity of HIF-1α TAD-N and TAD-C in a heterologous system.

The differential effect of DRB on CK2 activity and HIF-1 activity could be due to the fact that CK2 is already active in normoxia, so that it is inhibitable by DRB. On the other hand, HIF-1 is probably not active or to a very low extent in normoxia so that CK2 inhibition by DRB has no influence on its activity. Moreover, it may be possible that CK2 does not exert any influence on HIF-1 in normoxia since it is mainly located in the cytosol in these conditions. It has to be noted that hypoxia-induced increase in VEGF secretion is HIF-1-dependent but also AP-1-dependent and the effect, if any, of CK2 on AP-1 was not investigated in this work. The fact that apigenin but also TBB had a stronger effect than DRB on HIF-1 activity may indeed be due to an effect on HIF-1 transcriptional activity (as DRB) and a lower effect on HIF-1α stabilization. A recently published study indeed showed that a much higher concentration of apigenin inhibited HIF-1 activity through inhibition of HIF-1α stabilization.[57] However, as we worked with lower concentrations of apigenin, the results described here are probably more physiologic.

Two lines of hypotheses can be proposed to explain how CK2 could regulate HIF-1 activity. The first one involves a direct phosphorylation of HIF-1α by CK2. Phosphorylation of HIF-1α can be necessary, in addition to the nonhydroxylation of N803, for the recruitment of coactivators. As what was described for ERK1/2, phosphorylation of the coactivator itself[19] may also play a role. However, preliminary data shown in Figure 8 suggest that DRB does not affect CBP-HIF-1 interaction. Derepression of the inhibitory domain could be induced by conformational change due to the presence of one or more phosphate groups. Phosphorylation may also prevent the docking of FIH, the asparagine hydroxylase, and thus prevent the subsequent asparagine hydroxylation. Inhibition of phosphorylation, for example in the presence of DRB, would allow hydroxylation and inhibit HIF-1 activity. Two-hybrid screening in yeast has shown that CK2β has several binding partners, among them the collagen α-prolyl-4 hydroxylase,[58,59] but the mechanisms of their interaction as well as the functional consequence of the interaction are currently poorly understood, as is the question of whether CK2 would also interact with the HIF prolyl hydroxylase. The use of antibodies that could recognize the hydroxylated N803 would be helpful to confirm this hypothesis. One argument in favor of a direct phosphorylation of HIF-1α by CK2 is that DRB modified the electrophoretic pattern of HIF-1α. However, kinase assays using purified or immunoprecipitated CK2α and immunoprecipitated HIF-1α from hypoxic cells gave no indication of HIF-1α phosphorylation (data not shown). We also addressed the question whether HIF-1 would be directly phosphorylated by CK2. Gradin *et al.*[20] indicated that while T796 is phosphorylated *in vivo* and is a putative CK2 phosphorylation site, the TAD-C domain is not directly phosphorylated by CK2 *in vitro*. Computer analysis indicated that S551, S581, T700 and S787 in the human HIF-1α are also putative CK2 phosphorylation site with T700 having a higher score. In order to define if these putative sites may play a role in regulating the activity of HIF-1, each of these 3 amino acids have been point-mutated into alanine. The activity of each mutant has been assessed in a reporter assay in comparison with the wild-type HIF-1α and none of the mutant had a lower activity (data not shown). These results and the fact that CK2 inhibition inhibited both the TAD-N and the TAD-C activity suggest that this kinase modulates HIF-1 activity indirectly or that there are more than one phosphorylation site.

CK2 could also modulate HIF-1 activity through the phosphorylation of another protein interaction with HIF-1. CK2 is also known to phosphorylate p53, enhancing its stability and its activity.[60] In severe hypoxia or in anoxia, HIF-1α has been described to increase p53 stability[61] through an inhibition of its degradation by Mdm2.[62] This interaction between HIF-1α and p53 leads to an inhibition of HIF-1 activity because HIF-1α is targeted for degradation by Mdm2.[63] By contrast, in less severe hypoxia, hypoxia silences the p53 transactivation pathway by promoting a significant decrease in S392 phosphorylation on p53, which is a CK2 phosphorylation site.[64] It would thus result in increased HIF-1 activity. Whether CK2 is preferentially phosphorylating one (HIF-1α) rather than the other (p53) in hypoxia remains to be determined. On the other hand, it has to be noted that CK2 phosphorylation of p53 on T155 destabilizes the protein.[65] An inhibition of this process, for example by DRB, would thus result in more p53 and less HIF-1 activity, which is observed in this work.

CK2 also phosphorylates c-jun and DRB was shown to induce degradation of transiently expressed c-Jun in HeLa cells.[66] In this work, we also observed that DRB induced a marked decrease of c-Jun protein level, both in normoxia and in hypoxia (data not shown). Since HIF-1 and AP-1 are known to cooperate to upregulated the expression of several genes in hypoxia,[67–69] degradation of c-Jun by DRB may partly explain the decreased VEGF expression in hypoxia. However, it is unknown whether it would affect the HRE-driven expression of luciferase in a reporter system in hypoxia.

CK2 may regulate HIF-1 transcription factor by phosphorylating directly HIF-1α transactivation domains that contain multiple CK2 phosphorylation consensus sites or by phosphorylating cellular proteins involved in HIF-1 activation. Indeed, CK2β may interact and regulate different serine/threonine protein kinase such as Raf-1[70] and PKC.[71] Raf was already identified to play a role in the activation of the ERK MAP Kinase pathway and hence in HIF-1 activation in hypoxic conditions.[72] However, neither DRB nor apigenin affected the hypoxia-induced increase in ERK1/2 phosphorylation (data not shown).

Altogether, these results suggest for the first time that the ubiquitous serine/threonine kinase CK2 plays a crucial role in the post-translational regulation of HIF-1, mediating adaptation of the cell to hypoxia. An important task would be now to determine the mechanism of this CK2 function. In addition to the putative role of CK2 in regulating cell cycle checkpoint in cancer cells, its positive effect on HIF-1 activity could also positively contribute to the survival of these cells.

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