Casein kinase 1 regulates human hypoxia-inducible factor HIF-1

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Summary

Hypoxia-inducible factor 1 (HIF-1), a transcriptional activator that mediates cellular response to hypoxia and a promising target of anticancer therapy, is essential for adaptation to low oxygen conditions, embryogenesis and tumor progression. HIF-1 is a heterodimer of HIF-1α, expression of which is controlled by oxygen levels as well as by various oxygen-independent mechanisms, and HIF-1β (or ARNT), which is constitutively expressed. In this work, we investigate the phosphorylation of the N-terminal heterodimerization (PAS) domain of HIF-1α and identify Ser247 as a major site of in vitro modification by casein kinase 1δ (CK1δ). Mutation of this site to alanine, surprisingly, enhanced the transcriptional activity of HIF-1α, a result phenocopied by inhibition or small interfering RNA (siRNA)-mediated silencing of CK1δ under hypoxic conditions. Conversely, overexpression of CK1δ or phosphomimetic mutation of Ser247 to aspartate inhibited HIF-1α activity without affecting its stability or nuclear accumulation. Immunoprecipitation and in vitro binding experiments suggest that CK1-dependent phosphorylation of HIF-1α at Ser247 impairs its association with ARNT, a notion also supported by modeling the structure of the complex between HIF-1α and ARNT PAS-B domains. We suggest that modification of HIF-1α by CK1 represents a novel mechanism that controls the activity of HIF-1 during hypoxia by regulating the interaction between its two subunits.

Key words: HIF-1, Casein kinase, CK1, PAS, ARNT, Hypoxia

Introduction

Hypoxia-inducible factor 1 (HIF-1) is a transcriptional activator and the central component of hypoxia signaling, responding to changes in cellular oxygen levels. HIF-1 binds to regulatory DNA sequences called hypoxia-response elements (HRE) and controls the expression of genes involved in cell metabolism, erythropoiesis, angiogenesis, invasion and metastasis. It is, therefore, essential for adaptation to low oxygen conditions as well as for embryogenesis and tumor progression. HIF-1 is a heterodimer comprising the regulatory HIF-1α subunit and the constitutively expressed HIF-1β (or ARNT) subunit, both members of the basic helix-loop-helix (bHLH) Per-ARNT-Sim (PAS) family of transcription factors (Semenza, 2003; Semenza, 2007; Semenza, 2009). This family is characterized by the presence of the PAS dimerization domain that contains two conserved core repeats, PAS-A and PAS-B (Gu et al., 2000).

Under normal oxygen conditions, HIF-1α is continuously produced and destroyed, in a process involving hydroxylation, interaction with von Hippel-Lindau (VHL) protein, polyubiquitylation and subsequent proteasomal degradation (Schofield and Ratcliffe, 2005). Hydroxylation is mediated by three conserved prolyl hydroxylases (PHDs), the activity of which depends on the availability of oxygen, iron and 2-oxoglutarate (Semenza, 2001). Under hypoxic conditions, hydroxylation is impaired and HIF-1α is stabilized. HIF-1 transcriptional activity is also controlled by oxygen tension, as HIF-1α hydroxylation by FIH-1 (factor inhibiting HIF-1) at Asn803 compromises the association of its C-terminal transactivation domain (C-TAD) with the transcriptional co-activator CBP/p300 (Lancaster et al., 2004).

HIF-1α expression and activity are additionally regulated by oxygen-independent mechanisms. Activation of the phosphatidylinositol 3-kinase/AKT pathway leads to elevated translation of *HIF-1α* mRNA and HIF-1α production (Bardos and Ashcroft, 2005; Dery et al., 2005). Phosphorylation by glycogen synthase kinase 3 (GSK3) at three residues within the HIF-1α N-terminal transactivation domain (N-TAD) drives HIF-1α to VHL-independent proteasomal degradation (Flugel et al., 2007; Mottet et al., 2003). HIF-1α stability is also regulated by the molecular chaperone HSP90, inhibition of which induces HIF-1α degradation (Isaacs et al., 2002; Katschinski et al., 2004). RACK1 competes with HSP90 for binding to the HIF-1α N-terminal PAS-A domain. The RACK1–HIF-1α interaction is stabilized by SSAT1 and promotes the degradation of HIF-1α irrespective of oxygen levels (Baek et al., 2007; Liu et al., 2007).

The transcriptional activity of HIF-1α ultimately depends on its nuclear concentration and its ability to form a DNA-binding heterodimer with ARNT. Mitogen-activated protein kinase (MAPK) pathway activation potentiates HIF-1 transcriptional activity and HIF-1α is directly phosphorylated by p42/44 MAPK (Minet et al., 2000; Richard et al., 1999). HIF-1α phosphorylation by p42/44 MAPK occurs on residues Ser641 and Ser643 (Mylonis et al., 2006), and inhibition of this phosphorylation by mutagenesis, MAPK pathway inhibitors or flavonoid treatment impairs HIF-1α activity by triggering its nuclear export (Mylonis et al., 2006; Triantafyllou et al., 2008). This mechanism involves an atypical hydrophobic CRM1-dependent nuclear export signal in HIF-1α that becomes 'masked' when one or both of its proximal serine residues are phosphorylated by p42/44 MAPK (Mylonis et al., 2008). Phosphorylation by p42/44 MAPK, therefore, blocks HIF-1α export from the nucleus and ensures its efficient nuclear accumulation and full activation. The time, however, that HIF-1α spends in the nucleus and, ultimately, its activity also depend on its nuclear import rate. Recent studies have shown that HIF-1α physically interacts with multiple import receptors, including members of the importin-α family (Depping et al., 2008) and importins 4 and 7, which can mediate translocation of HIF-1α through nuclear pore complexes and its entry into the nucleoplasm (Chachami et al., 2009).

In our recent work (Mylonis et al., 2006), we have shown that an uncharacterized nuclear kinase targets the N-terminal part of HIF-1α (amino acids 1-251). In this study, we provide evidence that this kinase is casein kinase 1δ (CK1δ). CK1δ belongs to the CK1 protein kinase family, which consists of at least seven mammalian isoforms (α, β, γ1, γ2, γ3, δ and ε) and their splice variants. CK1 family members phosphorylate many substrates implicated in cell differentiation, proliferation and circadian rhythms (Knippschild et al., 2005). Our results show that phosphorylation of Ser247 in the HIF-1α PAS-B domain by CK1δ destabilizes the HIF-1α–ARNT complex, thereby diminishing its transcriptional activity. Therefore, regulation of HIF-1α–ARNT heterodimerization represents an additional means of oxygen-independent HIF-1 control, manipulation of which can have therapeutic importance in diseases involving HIF-1α.

Results

A nuclear kinase phosphorylates HIF-1α between amino acids 229 and 251

We previously reported that the N-terminal domain of HIF-1α (amino acids 1–251), which comprises the bHLH domain and most of the PAS domain, could be phosphorylated in vitro by a nuclear kinase distinct from MAPK (Mylonis et al., 2006). To investigate this further, smaller fragments of the N-terminal domain of HIF-1α (amino acids 1–71, 1–176, 72–176, 72–251 and 159–251) were expressed in bacteria as GST fusion proteins (Fig. 1A), purified and used as substrates in phosphorylation reactions catalyzed by a HeLa cell nuclear extract (Fig. 1B,C). This analysis mapped the phosphorylation site to fragment 159–251. Multiple sequence alignment analysis of this region of HIF-1α from several eukaryotes showed the presence of a smaller highly conserved area corresponding to amino acids 229–251 (Fig. 2A). Further analysis using programs DISPHOS (Iakoucheva et al., 2004) and ELM (Puntervoll et al., 2003) predicted that a conserved serine at position 247 (Ser247) was part of a CK1 site consensus sequence motif and might represent a target for phosphorylation. To experimentally check these predictions, region 230–251 was removed from the three previously tested HIF-1α fragments (Fig. 1A) and the resulting polypeptides (1–229, 72–229 and 159–229) were

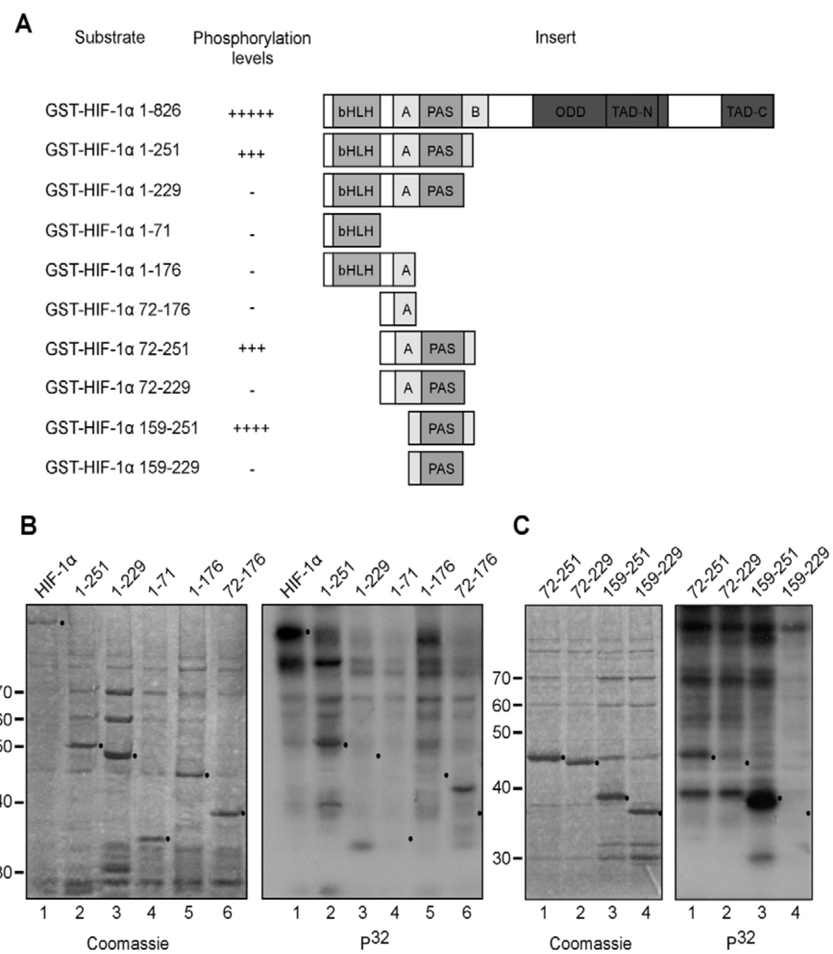

Fig. 1. A HeLa nuclear kinase phosphorylates HIF-1α between amino acids 229 and 251. (**A**) Schematic representation of HIF-1α and parts of its N-terminal domain phosphorylated by HeLa nuclear extracts. An approximate estimation of the relative phosphorylation levels, based on the results presented in B and C, is shown in the middle column. (**B**) The indicated GST-tagged HIF-1α fragments were phosphorylated by 2 μg HeLa nuclear protein extract and analyzed by SDS-PAGE followed by Coomassie Blue staining (left panel) and autoradiography (right panel; P^{32}). Dots indicate the positions of the recombinant GST-tagged HIF-1α fragments. Numbers on the left indicate the position of the corresponding molecular weight markers. (**C**) As in B, for additional HIF-1α fragments containing or lacking the 230–251 amino acid sequence.

subjected to phosphorylation. Indeed, deletion of the 230–251 sequence abolished phosphorylation in all three cases (Fig. 1B, compare lanes 2 and 3; Fig. 1C, compare lanes 1 and 2, 3 and 4). This showed that the major target of the nuclear kinase lies between amino acids 229 and 251, a region at the beginning of the conserved PAS-B motif and made Ser247 a prime candidate for phosphorylation.

CK1δ modifies HIF-1α at Ser247

To explore whether Ser247 is a target of modification, it was converted into alanine by site-directed mutagenesis (Fig. 2A). Protein fragments of HIF-1α carrying this mutation (1–251-S247A and 72–251-S247A) were used as substrates in phosphorylation assays along with their wild-type counterparts. As shown in Fig. 2B, mutation of Ser247 reduced dramatically the modification of the mutated fragments, suggesting that this residue is a major modification site in the N-terminal part of HIF-1α. We then checked whether this is also true in the context of full-length HIF-1α. To avoid MAPK-dependent modification, we introduced the S247A mutation into DM-HIF-1α, a double-mutant form of the protein that lacks the C-terminal MAPK-modification sites (Mylonis et al., 2006), and subjected it to phosphorylation along with the original (Ser247) DM form. As shown in Fig. 2C, the S247A mutation caused a substantial reduction in the phosphorylation levels of full-length DM-HIF-1α, confirming the major role of Ser247 in HIF-1α modification in vitro.

As already mentioned above, the sequence around Ser247 matches the consensus of a CK1 site motif. To test the possibility that the kinase activity targeting this site belongs to a CK1 isoform, we subjected wild-type full-length HIF-1α and its mutant HIF-1α S247A form to phosphorylation by recombinant CK1δ (Fig. 2D). The results show that HIF-1α is a substrate of CK1δ and indicate that a major target of this kinase is Ser247, because mutation of this residue results in a substantial reduction in the phosphorylation of HIF-1α (Fig. 2D). To further verify this, IC261, a CK1 inhibitor specific to isoforms δ and ε, was added to phosphorylation reactions containing as substrates full-length DM-HIF-1α and the HIF-1α 72–251 fragment (Fig. 2E). As shown in Fig. 2E (middle panels), IC261 inhibited phosphorylation of HIF-1α when CK1δ was used as the kinase source. When a HeLa cell nuclear extract was used as the kinase source, phosphorylation of HIF-1α was also effectively inhibited by IC261 (Fig. 2E, right panels), suggesting that the HeLa cell nuclear kinase activity targeting the N-terminal domain of HIF-1α corresponds to a CK1δ or CK1ε isoform. Taken

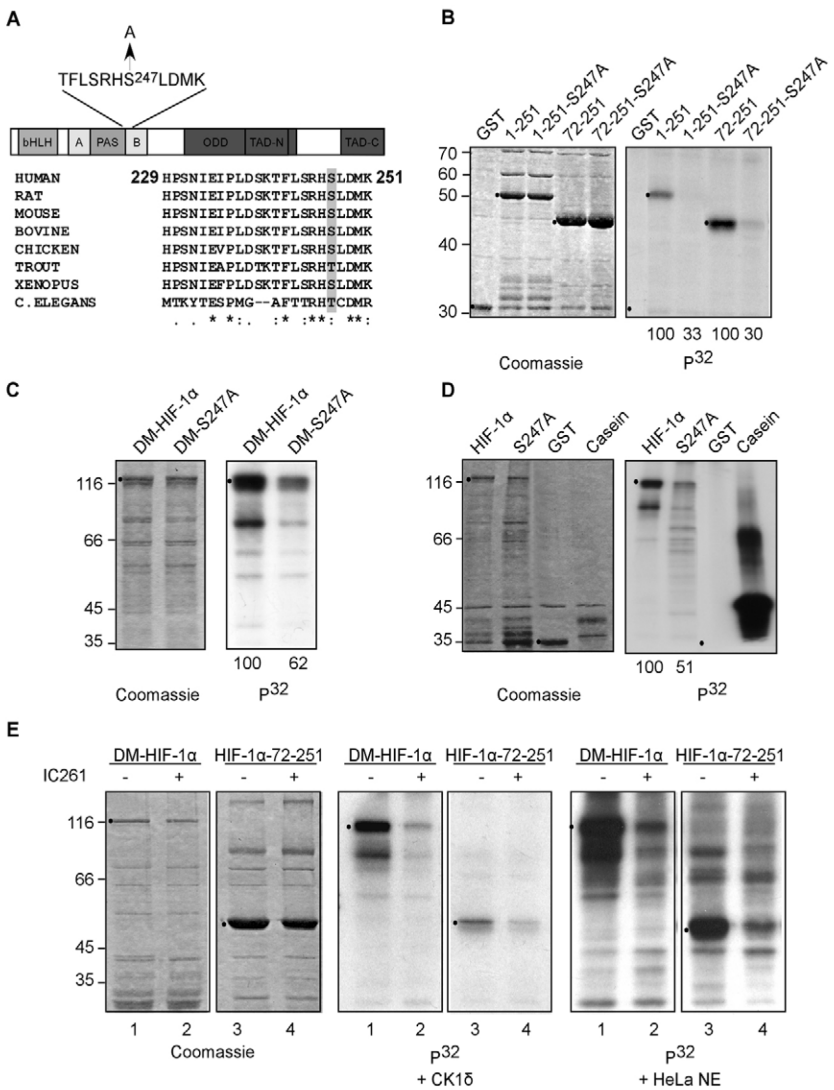

Fig. 2. Ser247 of HIF-1α is a target of CK1δ. (**A**) Schematic representation of human HIF-1α showing the position of Ser247, which is also highlighted in an alignment of the surrounding sequence (amino acids 229–251) with HIF-1α homologs from other species. (**B**) The indicated GST-tagged HIF-1α forms were phosphorylated by 2 μg HeLa nuclear protein extract and analyzed by SDS-PAGE followed by Coomassie Blue staining (left panel) and autoradiography (right panel; P^{32}). Dots indicate the positions of the recombinant GST-tagged fragments. The numbers underneath the lanes represent relative phosphorylation levels as average values of three independent experiments. (**C**) GST–HIF-1α forms mutated at serines 641 and 643 (DM-HIF-1α) or at serines 247, 641 and 643 (DM-S247A) were subjected to in vitro phosphorylation and analyzed as in B. (**D**) GST–HIF-1α, its S247A mutant form, GST and casein were subjected to in vitro phosphorylation by recombinant CK1δ and analyzed as in B. (**E**) GST–HIF-1α mutated at serines 641 and 643 (DM-HIF-1α) and GST–HIF-1α72–251 were phosphorylated in vitro by recombinant CK1δ (middle panel) or HeLa nuclear protein extract (right panel; HeLa NE) in the presence or absence of the CK1 inhibitor IC261 as indicated and analyzed as in B. Numbers on the left of the panels indicate the position of the corresponding molecular weight markers.

together, these results show that CK1δ modifies the N-terminal domain of HIF-1α in vitro at Ser247.

A closely related CK1 isoform, CK1ε, has been previously shown to sequentially target neighboring serine residues in mouse protein Per1 (Takano et al., 2004). It has also been reported that modification by CK1 often involves preceding phosphorylation of a residue N terminal to its target site within the consensus sequence S/T-x-x-S/T-x-x-S/T or the shorter S/T-x-x-S/T version (Knippschild et al., 2005). The location of Ser247 within HIF-1α conforms to this consensus ($\underline{T}^{241}FL\underline{S}^{244}RH\underline{S}^{247}$), suggesting a possible phosphorylation cascade. To explore the possibility that the conserved serine residue at position 244 (Ser244) of HIF-1α is also a target of CK1 or another priming kinase, we converted it into alanine. However, when mutant forms of HIF-1α carrying this mutation (S244A) were subjected to phosphorylation by nuclear extract or recombinant CK1δ, we could detect no significant differences compared with the corresponding wild-type forms (supplementary material Fig. S1A,B), suggesting that Ser244 is not a major modification site, at least in vitro (see also below).

Mutation of HIF-1α Ser247 to alanine stimulates HIF-1 transcriptional activity

To study the role of HIF-1α Ser247 phosphorylation, wild-type HIF-1α and the non-phosphorylatable S247A mutant form were expressed as GFP fusion proteins in HEK293T cells. Western blot analysis showed that wild-type GFP–HIF-1α and its mutant S247A form were both expressed, with the mutant form having slightly lower expression levels (supplementary material Fig. S2A). Similar expression and nuclear accumulation was also confirmed by fluorescence microscopy (supplementary material Fig. S2B). Surprisingly, using a luciferase reporter gene assay, the mutant S247A form demonstrated a significantly greater HRE-dependent transcriptional activity compared with that of wild-type HIF-1α (Fig. 3A), suggesting that mutation of Ser247 is not detrimental but rather beneficial to the function of HIF-1α.

To confirm the effect of the mutation by an additional human-cell-free but in vivo method, the transcriptional activity of HIF-1α and its S247A mutant was measured using a previously developed assay system in yeast (Braliou et al., 2006). As shown in Fig. 3B, the mutant S247A form exhibited a significant increase in its transcriptional activity, in good agreement with the results obtained in human cells. Taken together, our data suggest that lack of phosphorylation of Ser247 positively affects a step in HIF-1α activation that is not linked to HIF-1α protein expression or localization.

To test whether modification of Ser244, which does not detectably take place in vitro (see above), might, nevertheless, have a similar effect to Ser247 phosphorylation, the activity of the HIF-1α S244A mutant form was measured in both human and yeast cells, as described above. Unlike S247A, in human cells the mutant S244A form exhibited lower transcriptional activity than the wild-type form, whereas in yeast cells its activity was similar to that of the wild-type form (supplementary material Fig. S1C,D). Therefore, even if Ser244 is modified in vivo, it does not seem to play the same role as Ser247 (see also Discussion).

Inhibition of CK1 stimulates HIF-1 transcriptional activity

The fact that Ser247 of HIF-1α is a target of CK1 in vitro and that its mutation into alanine affects the function of HIF-1α in both yeast and mammalian cells indicates that CK1 might play a role in the regulation of HIF-1, which is physiologically induced under

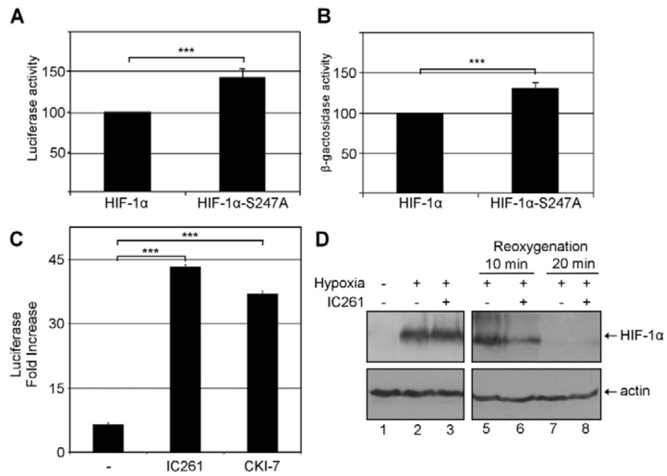

Fig. 3. Mutation of Ser247 into alanine or inhibition of CK1 stimulates the transcriptional activity of HIF-1α. (**A**) Determination of HIF-1 transcriptional activity 24 hours post-transfection in HEK293T cells expressing GFP-tagged wild-type HIF-1α or the HIF-1α S247A mutant form as indicated. Results are shown as percent of activity in relation to wild-type HIF-1α and represent the mean of three independent experiments performed in triplicate (± s.e.m.). (**B**) Determination of HIF-1 transcriptional activity in yeast cells expressing inducible wild-type HIF-1α or its S247A mutant form together with human ARNT and β-galactosidase as a reporter under the control of an HRE-containing promoter. Results are shown as in A. (**C**) Determination of HIF-1 transcriptional activity 24 hours post-transfection in HeLa cells incubated for 16 hours under normoxia or hypoxia (1% O_2) in the presence or absence of the CK1 inhibitors IC261 (2 μM) or CKI-7 (10 μM). Results are shown as fold increase in relation to the corresponding normoxic conditions and represent the mean of three independent experiments performed in triplicate (± s.e.m.). (**D**) Western blot analysis of extracts from HeLa cells incubated for 6 hours in normal or hypoxic (1% O_2) conditions and treated for 4 hours with the CK1 inhibitor IC261 (2 μM). HeLa cells were lysed either right at the end of the hypoxic incubation period (left panels) or after re-oxygenation for 10 or 20 minutes as indicated (right panels). HIF-1α (upper panels) and β-actin (lower panels; loading control) were detected by rabbit polyclonal or mouse monoclonal antibodies, respectively.

hypoxic conditions. To test this hypothesis, human cervical cancer HeLa cells were subjected to hypoxia (1% O_2) in the absence or presence of the specific CK1δ and CK1ε inhibitor IC261 (2 μM) or the more general CK1 inhibitor CKI-7 (10 μM) (Chijiwa et al., 1989; Mashhoon et al., 2000). As shown in Fig. 3C, the transcriptional activity of endogenous HIF-1 was drastically enhanced when hypoxically treated cells were also exposed to either one of the two CK1 inhibitors. Western blot (Fig. 3D, left panels) and immunofluorescence (supplementary material Fig. S2C) analysis of HeLa cells incubated under hypoxia showed no significant changes in the expression levels and nuclear localization of endogenous HIF-1α when CK1 was concomitantly inhibited by IC261. Furthermore, the stability of hypoxically induced HIF-1α was not increased upon re-oxygenation, when the cells were also simultaneously treated with IC261, and was rapidly degraded (Fig. 3D, right panels).

To confirm the effect of CK1 inhibition on HIF-1 activity, we tested additional cell lines and endogenous HIF-1 target genes. As shown in Fig. 4A, when human hepatoma HRG1 cells [HepG2 cells stably transfected with a hypoxia-responsive luciferase reporter gene (Wagner et al., 2008)] were subjected to hypoxia, the transcriptional activity of endogenous HIF-1 was significantly

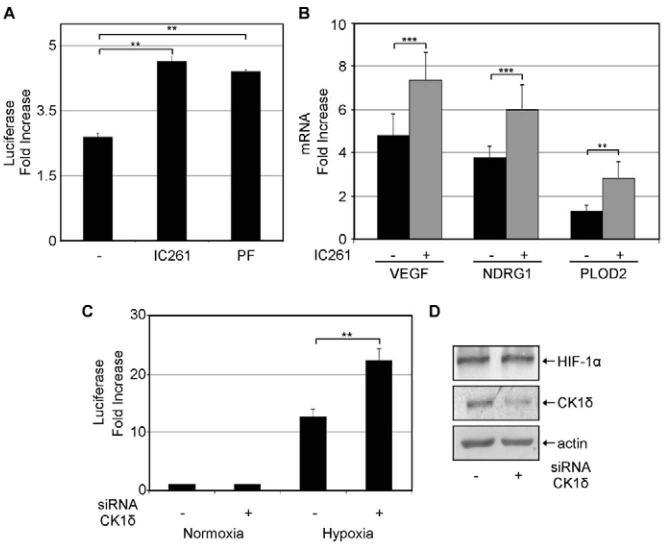

Fig. 4. Inhibition or silencing of CK1δ stimulates HIF-1 transcriptional activity. (**A**) Determination of HIF-1 transcriptional activity in HRG1 cells incubated for 6 hours under normoxia or hypoxia (1% O_2) in the presence or absence of the CK1 inhibitors IC261 (2 μM) or PF670462 (0.5 μM). Results are shown as fold increase in relation to the corresponding normoxic conditions and represent the mean of two independent experiments performed in triplicate (± s.e.m.). (**B**) Determination of VEGF, NDRG1 and PLOD2 mRNA levels by quantitative real-time PCR in hBSMCs incubated for 16 hours under normoxia or hypoxia (1% O_2) in the presence (gray bars) or absence (black bars) of the CK1 inhibitor IC261 (2 μM). Results are shown as in A. (**C**) Determination of HIF-1 transcriptional activity 48 hours after transfecting Huh7 cells with CK1δ siRNA (10 nM) or scrambled siRNA (10 nM) and reporter plasmids. Cells were incubated in normoxia or hypoxia for 4 hours before collection and lysis. Values are expressed as in A. (**D**) Western blot analysis of extracts from Huh7 cells treated with siRNA as in C and exposed to hypoxia, using anti-HIF-1α, anti-CK1δ and anti-actin antibodies as indicated.

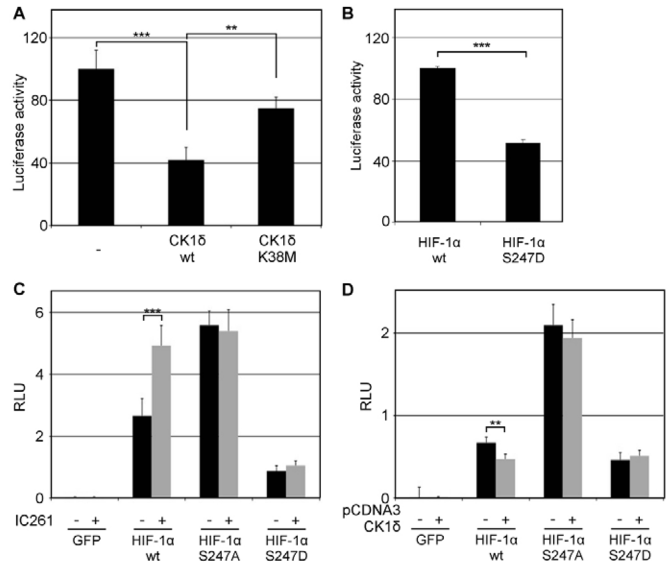

Fig. 5. Overexpression of CK1δ or the phosphomimetic mutation S247D decreases the transcriptional activity of HIF-1α. (**A**) Determination of HIF-1 transcriptional activity 24 hours post-transfection of HEK293T cells with reporter plasmids and pCDNA3.1-CK1δ or pCDNA3.1-CK1δ-K38M, incubated under hypoxia for 16 hours. Values are expressed in relation to the value obtained for the empty pCDNA3 vector and represent the mean of two independent experiments performed in triplicate (± s.e.m.). (**B**) Determination of HIF-1 transcriptional activity 24 hours post-transfection of HEK293T cells expressing GFP-tagged wild-type HIF-1α or the HIF-1α S247D mutant form as indicated. Results are shown as percent of activity in relation to wild-type HIF-1α and represent the mean of three independent experiments performed in triplicate (± s.e.m.). (**C**) Determination of HIF-1 transcriptional activity 24 hours post-transfection of HEK293T cells expressing GFP-tagged wild-type HIF-1α or its mutant forms as indicated. Cells were incubated for 16 hours in the presence (gray bars) or absence (black bars) of IC261 (2 μM). Results are shown as relative luciferase units (RLU) and represent the mean of three independent experiments performed in triplicate (± s.e.m.). (**D**) Determination of HIF-1 transcriptional activity as in C in HEK293T cells co-transfected with the pCDNA3-CK1δ plasmid (gray bars) or its corresponding empty pCDNA3 vector (black bars) as indicated.

increased by both IC261 (2 μM) and the recently developed CK1δ and CK1ε inhibitor PF-670462 (0.5 μM) (Badura et al., 2007). We then examined the expression levels of the endogenous hypoxia-induced HIF-1 targets VEGF (vascular endothelial growth factor), NDRG1 (N-myc downstream-regulated gene 1) (Ellen et al., 2008) and PLOD2 (procollagen lysyl-hydroxylase 2) (Hofbauer et al., 2003) in primary, non-transformed human bronchial smooth muscle cells (hBSMCs). Quantification of mRNA levels by real-time PCR showed induction of the genes upon hypoxic treatment of the cells (Fig. 4B). When exposure to hypoxia was performed in the presence of IC261, the expression levels of VEGF, NDRG1 and PLOD2 were all significantly increased (Fig. 4B).

To finally show that the kinase responsible for HIF-1 inhibition in vivo is indeed CK1δ, we specifically suppressed its expression in Huh7 hepatoma cells by small interfering RNA (siRNA)-mediated silencing. As shown in Fig. 4D, treatment of these cells with *CK1δ* siRNA under hypoxic conditions substantially reduced the expression of CK1δ (compared with cells treated with control siRNA), but did not affect the expression levels of endogenous HIF-1α. Nevertheless, suppression of CK1δ resulted in an approximately twofold increase in HIF-1 transcriptional activity under the same hypoxic conditions (Fig. 4C).

Taken together, these data show that inhibition of CK1 activity stimulates HIF-1 activity under hypoxia. Combined with the observed higher activity of the S247A mutant, this suggests that direct modification of HIF-1α at Ser247 by CK1 has a negative impact on its function.

Overexpression of CK1δ or phosphomimetic mutation of Ser247 to aspartate inhibits HIF-1 transcriptional activity

To confirm that CK1 negatively affects HIF-1 activity, CK1δ or its catalytically inactive mutant form, CK1δK38M, were overexpressed in HEK293T cells that were subsequently incubated under hypoxia. As shown in Fig. 5A, the hypoxic HRE-dependent transcriptional activity of HIF-1 was reduced upon CK1δ overexpression. The extent of inhibition was much lower when the mutant kinase form was overexpressed (Fig. 5A), suggesting that the enzymatic activity of CK1δ is responsible for the downregulation of HIF-1 activity. The weak inhibition caused by the mutant form of CK1δ might be due to formation of 'unproductive' kinase–substrate complexes that interfere with the function of HIF-1α despite the lack of phosphorylation.

Another way to positively demonstrate the role of Ser247 phosphorylation was to convert this serine to aspartate (S247D), the negative charge of which could potentially mimic the charge of phosphoserine. Wild-type HIF-1α and the phosphomimetic (or pseudophosphorylated) mutant HIF-1α S247D form were expressed as GFP fusions in HEK293T cells. The transcriptional activity of GFP–HIF-1α S247D was significantly lower than that of the wild-type form (Fig. 5B). This reduction was not due to instability or mislocalization of the mutant protein, as expression levels of both wild-type HIF-1α and HIF-1α S247D were shown by western blot analysis to be similar (supplementary material Fig. 2D), and both forms of HIF-1α were nuclear (supplementary material Fig. 2E). Thus, both loss-of-function and gain-of-function experiments show that phosphorylation of Ser247 by CK1δ limits the activation of HIF-1 caused by hypoxia.

The effect of CK1 on HIF-1 activity is predominantly mediated by Ser247 modification

The data described so far show that both inhibition of CK1 and the HIF-1α S247A mutation have positive effects on HIF-1 activity, whereas both overexpression of CK1 and the HIF-1α S247D mutation are negative factors. However, they do not formally exclude the possibility that the CK1 effect is unrelated or only partially linked to the modification of Ser247. To test this possibility, the wild-type, the unphosphorylatable S247A mutant and the pseudophosphorylated S247D mutant forms of HIF-1α were expressed as GFP fusions in HEK293T cells, and HIF-1 transcriptional activity was determined under conditions that either inhibited (incubation with IC261) (Fig. 5C) or stimulated (overexpression of CK1δ) (Fig. 5D) CK1 catalytic activity. In both experiments, GFP–HIF-1αS247A and GFP–HIF-1αS247D exhibited higher and lower, respectively, activity compared with the wild-type form under control conditions. Furthermore, the wild-type form responded to inhibition or overexpression of CK1δ by exhibiting significantly higher or lower activity, respectively, exactly reproducing the behavior of endogenous hypoxically induced HIF-1α. However, the transcriptional activities of the mutant S247A and S247D forms were not significantly affected by either inhibiting (Fig. 5C) or stimulating (Fig. 5D) CK1 activity. These results strongly suggest that regulation of HIF-1α by CK1δ in vivo involves solely or predominantly Ser247.

Phosphorylation of HIF-1α by CK1 inhibits its heterodimerization with ARNT

As the observed changes of HIF-1α activity when phosphorylation of Ser247 by CK1 is perturbed cannot be attributed to its altered expression, stability or nuclear localization, the affected step in HIF-1α activation must lie downstream of its nuclear translocation, that is, it might correspond to its ability to form an active complex with ARNT. This is actually also suggested by the fact that Ser247 lies inside the PAS heterodimerization domain.

To investigate this possibility, HIF-1α was induced in HeLa cells either by treatment with the PHD inhibitor DMOG (1 mM) at normoxia or by hypoxia (1% O_2). Following treatment with or without the CK1 inhibitor IC261, HIF-1α was immunoprecipitated from total cell extracts. In both DMOG and hypoxia cases, analysis of the immunoprecipitates revealed much stronger association of ARNT with HIF-1α when cells were treated with IC261 (Fig. 6A, lanes 3 and 6) compared with DMOG or hypoxia alone (Fig. 6A, lanes 2 and 5, respectively). It can, therefore, be concluded that HIF-1α phosphorylation by CK1 impairs HIF-1 activity by preventing HIF-1α interaction with ARNT. To confirm again that the CK1 effect is due to changes of Ser247, GFP–HIF-1α or its mutant S247A and S247D forms were immunoprecipitated

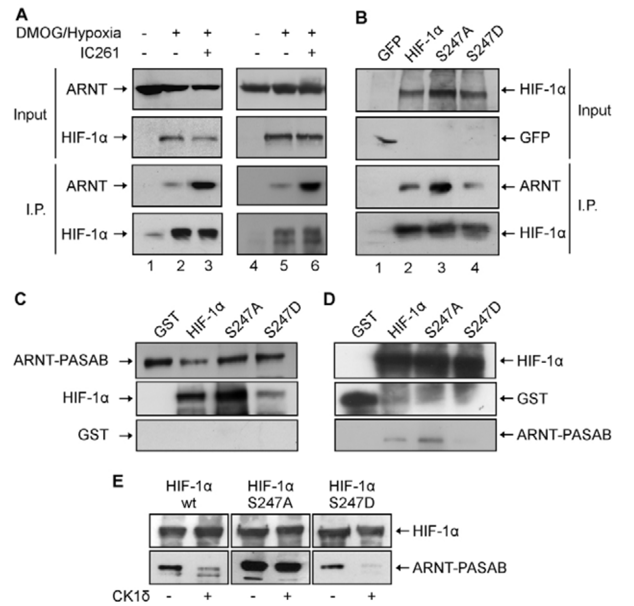

Fig. 6. Inhibition of CK1 and the Ser247 status interfere with the interaction between HIF-1α and ARNT in vivo and in vitro. (**A**) HeLa cells were incubated for 4 hours with 1 mM DMOG (left panels) or under hypoxia (1% O_2) (right panels) in combination with 2 μM IC261 as indicated. Cells were lysed and subjected to immunoprecipitation with an anti-HIF-1α antibody. Total cell extract (input) and precipitated proteins (IP) were analyzed by western blot using anti-ARNT and anti-HIF-1α antibodies as indicated. Only the relevant parts of the blots are shown. (**B**) HEK293T cells expressing GFP or the indicated GFP-tagged forms of HIF-1α were lysed and subjected to immunoprecipitation with an anti-HIF-1α antibody. Total cell extract (input) and precipitated proteins (IP) were analyzed by western blot using anti-GFP, anti-ARNT and anti-HIF-1α antibodies as indicated. Only the relevant parts of the blots are shown. (**C**) Purified soluble GST, GST–HIF-1α or each of its two S247A and S247D mutant forms were incubated with His-tagged ARNT bHLH–PAS immobilized on Ni–NTA–agarose beads. Bound proteins were analyzed by SDS-PAGE and western blot using antibodies against the His-tag, HIF-1α or GST as indicated. (**D**) As in C, but purified soluble His-tagged ARNT bHLH–PAS was incubated with GST, GST–HIF-1α or each of its two mutant S247A and S247D forms immobilized on GSH–sepharose beads. (**E**) As in D, but GST–HIF-1α or each of its two mutant S247A and S247D forms were incubated in the absence or presence of recombinant CK1δ for 1 hour at 30°C prior to treatment with His-tagged ARNT bHLH–PAS.

from cells transiently expressing them. Analysis of the immunoprecipitates revealed stronger association of ARNT with GFP–HIF-1αS247A and weaker association with GFP–HIF-1α S247D compared with the wild-type form (Fig. 6B).

To further establish the role of Ser247 phosphorylation in HIF-1 heterodimerization, we tested in vitro the binding (by pull-down assays) of recombinant GST-tagged wild-type HIF-1α or its mutant S247A and S247D forms to His-tagged ARNT-bHLH-PAS, the N-terminal part of ARNT (amino acids 1–474) comprising the bHLH and PAS domains. All forms of HIF-1α, but not GST alone, could bind to immobilized ARNT bHLH–PAS (Fig. 6C). However, binding of the pseudophosphorylated HIF-1α S247D form was substantially weaker than that of either wild-type HIF-1α or the mutant HIF-1α S247A form (Fig. 6C). To confirm this result, we performed the reverse experiment, that is, we checked binding of ARNT bHLH–PAS to immobilized GST-tagged forms of HIF-1α (Fig. 6D). Again, association of ARNT bHLH–PAS with the phosphomimetic HIF-1α S247D mutant was scarcely detectable and substantially weaker than with the wild-type or mutant S247A forms (Fig. 6D). To test directly the involvement of CK1 in the interaction between HIF-1α and ARNT, the last experiment was repeated, but the immobilized forms of HIF-1α were first incubated with recombinant CK1δ and ATP before they were exposed to ARNT bHLH–PAS. As shown in Fig. 6E, treatment with CK1δ indeed inhibited the interaction between wild-type HIF-1α and ARNT bHLH–PAS, but did not detectably affect the corresponding interaction of HIF-1α S247A. Treatment with CK1δ further reduced the already weak association of HIF-1α S247D with ARNT bHLH–PAS, suggesting that additional sites might become accessible to CK1δ in vitro. The results of the in vivo and in vitro association experiments, considered together, strongly suggest that phosphorylation of Ser247 by CK1 regulates the formation of a functional HIF-1α–ARNT heterodimer.

A structural model explains the involvement of Ser247 phosphorylation in HIF-1α–ARNT heterodimerization

The three-dimensional structure of the HIF-1α PAS-B domain is not known. However, its high sequence identity (77%) with the PAS-B domain of HIF-2α allowed us to reliably model it, as judged by several well-established structure validity criteria (see Materials and Methods). The model structure of HIF-1α shows that the sidechain of Ser247 is solvent exposed and easily accessible to a kinase (Fig. 7A). Interestingly, this residue is highly conserved in the family of PAS domains, occasionally being replaced by a threonine or an aspartic acid residue. It is also noteworthy that similar highly conserved amino acids in this region, such as Thr241 and Ser244, are located in the central part of a β-sheet, engaged in strong hydrogen bonding crucial for the structural integrity of the protein and deeply buried in the hydrophobic core of the protein. Therefore, it is highly unlikely that the sidechains of these residues could be readily accessible to a kinase, in agreement with our experimental evidence concerning Ser244 (see above). By contrast, the sidechain of Ser247 (or of the equivalent residue in that position) is free of any interactions in all examined known structures of PAS domains. Furthermore, Ser247 lies very close to the interface of the HIF-1α–ARNT PAS-B complex (Fig. 7B). Its phosphorylation can substantially alter the surface-charge distribution of HIF-1α PAS-B, close to the cavity that forms part of the HIF-1α–ARNT complex interface (Fig. 7C,D).

When combined with recently published data on the structural basis of HIF-2α–ARNT heterodimerization, our model provides clues to the experimentally observed destabilization of the HIF-1α–ARNT heterodimer upon Ser247 phosphorylation. Recent reports of an NMR-guided model of the human HIF-2α–ARNT PAS-B heterodimer and its crystal structure (Card et al., 2005; Scheuermann et al., 2009) revealed the role of the interfacial ionic interaction between HIF-2α Arg247 (which corresponds to Arg245 in HIF-1α) and ARNT Glu362 sidechains in the formation of the complex. The wild-type monomers have comparatively low affinity for each other, which improves substantially upon combination of ARNT E362R and HIF-2α R247E mutations. It has been suggested that the salt bridge formed between HIF-2α Arg247 and ARNT Glu362 is dampened by the adjacent positive charge on ARNT Arg379. The complementary mutations enhanced the positive charge on the ARNT side of the complex, thereby strengthening the ionic interaction present in the wild-type heterodimer. Because Ser247 is found, according to our model of the HIF-1α–ARNT complex, in the vicinity of the interfacial ionic interaction between HIF-1α Arg245 and ARNT Glu362 (Fig. 7E), it is reasonable to suggest, by analogy, that the strong negative charge introduced by Ser247 phosphorylation dampens the positive charge of Arg245 on the HIF-1α side of the complex and destabilizes the heterodimer (Fig. 7F). A similar effect has been indeed observed experimentally in the case of the HIF-2α–ARNT complex, with the affinity of the HIF-2α R247E mutant for wild-type ARNT being considerably lower than that of the native HIF-2α protein (Scheuermann et al., 2009).

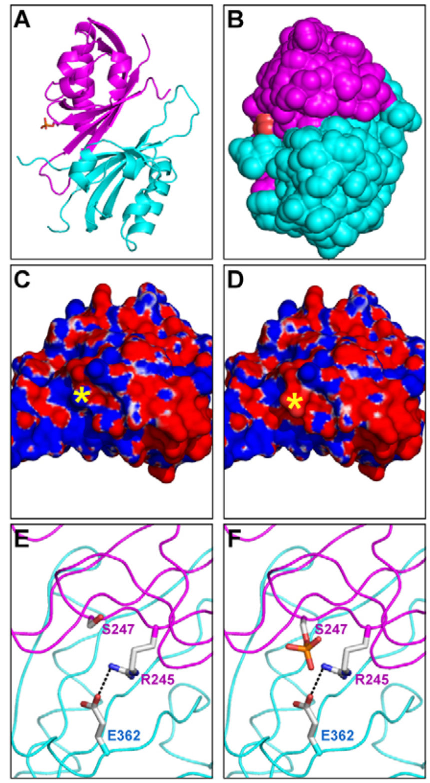

Fig. 7. Molecular modeling of the complex between the HIF-1α and ARNT PAS-B domains reveals the significance of Ser247 phosphorylation. (**A**) Ribbon diagram of the model structure of the heterodimer formed between the PAS-B domains of HIF-1α (magenta) and ARNT (blue), based on existing structural data for the HIF-2α–ARNT PAS-B complex. The position of the exposed Ser247 is shown in its phosphorylated form (orange). (**B**) A space-filling model of the structure of the HIF-1α–ARNT PAS-B heterodimer. Color scheme as in A. (**C**) Surface electrostatic potential of the monomeric HIF-1α PAS-B domain, calculated by APBS and displayed on the structure using a color gradient ranging from negative (red = –4 kT) to neutral (white) to positive (blue = +4 kT). The yellow asterisk close to the cavity that forms part of the HIF-1α–ARNT complex interface shows the position of Ser247. (**D**) As in C, but the sidechain of Ser247 is substituted in the model by its phosphorylated form. (**E**) Model structure of the HIF-1α (magenta) and ARNT (blue) PAS-B heterodimer using a tube representation for the protein backbone and stick diagrams for the sidechains of residues Arg245 (on HIF-1α) and Glu362 (on ARNT). which form a salt bridge previously shown to be crucial for the stabilization of the heterodimer. The sidechain of the adjacent Ser247 in HIF-1α is also indicated. (**F**) As in E, with the sidechain of Ser247 phosphorylated.

Inhibition of CK1 enhances cell proliferation under hypoxic conditions

The activity of HIF-1 is very important for survival and proliferation of cells under hypoxic conditions. Because we have shown that

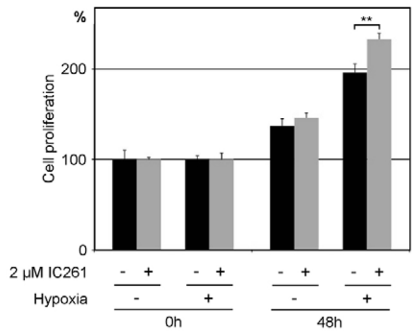

Fig. 8. CK1 inhibition promotes proliferation of Huh7 cells under hypoxia. Digitized graph of Huh7 cell proliferation under normoxic or hypoxic (1% O_2) conditions and in the presence (gray bars) or absence (black bars) of 2 μM of IC261. Data represent the mean (± s.e.m.) of two independent experiments performed in triplicate and are expressed as percent of the initial number of cells at time zero.

CK1 negatively affects HIF-1, we checked whether inhibition of CK1 influences the viability of Huh7 hepatoma cells after prolonged (48 hours) incubation under normoxic or hypoxic conditions. As shown in Fig. 8, treatment of the cells with 2 μM IC261 did not significantly affect their growth rate under normal oxygen concentration conditions. However, at 1% oxygen, IC261 caused a small but statistically significant increase in cell proliferation, in agreement with its positive effect on HIF-1 activity. It is, therefore, possible that modulation of CK1 activity can influence important HIF-1-dependent processes, such as growth of cancer cells, under low-oxygen conditions.

Discussion

Revealing additional oxygen-independent mechanisms of HIF-1 regulation is not only important for understanding its biological function but, given its involvement in ischemia and cancer, also essential for developing new and targeted therapeutic intervention methods. In this report, we have identified and molecularly dissected a novel regulatory mechanism that involves CK1, Ser247 of HIF-1α and the formation of the HIF-1α–ARNT heterodimer. Our conclusion that phosphorylation of HIF-1α by CK1δ at Ser247 impairs heterodimer formation and, thus, inhibits HIF-1 activity is based on the following observations. First, CK1δ (or closely related CK1ε isoform) is the nuclear kinase that modifies the PAS domain of HIF-1α, targeting predominantly Ser247, in vitro. Second, inhibition of CK1δ and CK1ε does not affect the stability or localization of HIF-1α, but increases its association with ARNT and its transcriptional activity under hypoxia in vivo. Third, overexpression of CK1δ inhibits, whereas its silencing stimulates the hypoxic activity of HIF-1α. Fourth, inhibition of Ser247 phosphorylation (by converting it into Ala247) increases the association of HIF-1α with ARNT and stimulates the activity of HIF-1α, in both human and yeast cells (the latter containing ARNT as the only other human protein), without increasing its expression or nuclear accumulation. Fifth, mimicking Ser247 phosphorylation (by converting it into Asp247) impairs the association of HIF-1α with ARNT and inhibits HIF-1α activity in vivo, again without affecting its stability or localization. Sixth, structural modeling of the HIF-1α–ARNT PAS-B domain complex reveals that Ser247 phosphorylation is indeed feasible and is in a perfect position to interfere with complex formation.

The observation that an isoform of CK1 can modify and regulate HIF-1α is made for the first time, despite the fact that CK1 family members have long been known to phosphorylate substrates involved in many cellular processes, such as differentiation, proliferation and circadian control (Knippschild et al., 2005). CK1 activity has been associated with pleiotropic effects on its targets and the pathways they are involved in. Its role in the regulation of nucleocytoplasmic transport and the stability of proteins participating in the circadian clock pathway has been well established (Akashi et al., 2002; Knippschild et al., 2005; Lee et al., 2001). However, its function in the regulation of pathways associated with cell proliferation, such as those involving p53 or Wnt, can have positive or negative effects depending on cell type and conditions (Knippschild et al., 2005; Price, 2006). Our work identifies HIF-1 and the cellular response to hypoxia as additional targets of CK1, which, in this case, appears to function negatively by limiting the formation of a functional HIF-1 heterodimer.

The target of CK1 in the case of HIF-1α is the PAS domain, Ser247 being the predominant (if not the only) modified residue. The PAS domain is an evolutionarily conserved motif composed of two core repeats (PAS-A and PAS-B) spaced by a linker sequence of variable length. It is usually present in proteins that act as integrators of various stimuli (polycyclic aromatic hydrocarbons, cellular rhythms or hypoxic response). The activity of these proteins is often linked to heterodimerization through their PAS domains and to interactions with multiple cofactors that regulate their activity (Gu et al., 2000). Concerning HIF-1α, it has long been established that it associates with ARNT through its bHLH and PAS domains to form a functional HIF-1 heterodimer (Gradin et al., 1996). Furthermore, the HIF-1α PAS domain has been shown to be involved in the oxygen-independent regulation of HIF-1α stability by means of its interactions with HSP90 (Isaacs et al., 2002; Katschinski et al., 2004; Minet et al., 1999), ARNT (Isaacs et al., 2004), RACK1 (Liu et al., 2007) and SSAT1 (Baek et al., 2007), the latter two binding to the PAS-A motif. By contrast, Ser247 is located at the beginning of the PAS-B conserved motif and its phosphorylation status appears to not affect HIF-1α stability, but rather its association with ARNT. According to detailed studies (Chapman-Smith et al., 2004), the PAS-B subdomain of HIF-1α has little effect on binding to the HRE DNA. However, as HIF-1α–ARNT heterodimerization is an obligatory step before HRE recognition (Kallio et al., 1997), inhibition of heterodimerization impairs DNA binding and should result in loss of HIF-1 transcriptional activity, as we indeed observe upon Ser247 phosphorylation by CK1δ. The PAS domain plays no role in the interactions of HIF-1α with the PHDs, VHL, FIH-1 or CBP/p300, which are mediated by its ODD and C-TAD domains.

Although both our biochemical and structural results clearly implicate Ser247 as a crucial phosphorylation target, they do not completely exclude the possibility that additional HIF-1α residues involved in its heterodimerization process are modified by CK1δ. In fact, the effect of CK1δ inhibition on HIF-1 activity is more pronounced than the effect of the S247A mutation. It is, however, possible that, in vitro, CK1δ might modify sites that are normally not accessible in vivo. This is supported by our observation that treatment of the HIF-1α S247D mutant with CK1δ in vitro further diminishes its ability to interact with ARNT, whereas the same mutant remains virtually unaffected when the activity of CK1 is perturbed in vivo. Alternatively, CK1δ might also target the second heterodimerization partner, ARNT. Although this remains to be shown, alignment of the PAS-B domains from HIF-1α and

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ARNT (Taylor and Zhulin, 1999) shows that the ARNT residue that corresponds to HIF-1α Ser247 is Asn368, making similar modification of ARNT less likely.

The CK1 canonical substrate consensus sequence, S/T(P)-X_{1-2}-S/T, which is also found in HIF-1α (^{244}SRHS^{247}), indicates that modification by CK1 might require the preceding phosphorylation of a residue N-terminal of the target site. However, we were unable to obtain data supporting phosphorylation of Ser244 in vitro. It appears that, also in vivo, modification of Ser244 is not a prerequisite for Ser247 phosphorylation, because the effects of their mutations on HIF-1 activity are opposite. According to our structural model, Ser244 is buried in the protein core and is engaged in strong hydrogen bonding crucial for the structural integrity of the protein. Therefore, its hydroxyl group is not only inaccessible for modification but also its removal might destabilize HIF-1α, thus causing the observed reduction in its activity. As previously reported for other proteins (Knippschild et al., 2005), HIF-1α recognition by CK1 might depend on the presence of a non-canonical motif or on the tertiary structure of PAS-B.

HIF-1α not only plays a seminal role in the hypoxia-response pathway, but also is associated with both physiological and pathogenic conditions, including embryogenesis, ischemic tissue survival and tumor progression. As its dimerization with ARNT is an obligatory step for HIF-1 activation, the CK1-dependent mechanism we have described is very important for controlling HIF-1 activity, under both hypoxia and normoxia, and opens up new ways of investigating HIF-1α and interfering therapeutically with its function. It also raises questions concerning the extent and significance of CK1 involvement in the hypoxic response and related pathological conditions. Because CK1 family members are widely distributed constitutively expressed enzymes and because the mechanisms of controlling their activity in the mammalian cell, especially in response to external stimuli, are poorly understood (Knippschild et al., 2005), these issues cannot be easily addressed. However, our findings bring forward several interesting hypotheses. As HIF-1α is required for the proliferation of cancer cells (Semenza, 2009), CK1, by negatively affecting HIF-1 activity, could possibly have anti-proliferative effects, a fact also suggested by our cell proliferation experiment. This is consistent with observations concerning the effect of CK1 isoforms on the activity of the p53 tumor suppressor protein. Upon cell stress, CK1 phosphorylates p53 in its N-terminal region, weakens the interaction with MDM2 and, therefore, stabilizes and activates p53 function (Alsheich-Bartok et al., 2008; Knippschild et al., 1997; Sakaguchi et al., 2000).

An alternative function of the CK1-dependent phosphorylation of HIF-1α might be related to the ability of both HIF-1α and ARNT to interact not only with each other, but also with other proteins. ARNT also forms a complex with the aryl hydrocarbon receptor (AhR) (Beischlag et al., 2008), whereas HIF-1α can bind to the Myc-associated protein X (MAX) or to Sp1, antagonizing in both cases c-Myc activity (Dang et al., 2008; Gordan et al., 2007; Huang, 2008; Koshiji et al., 2005). Therefore, by weakening the affinity of HIF-1α for ARNT, CK1 could make both subunits more available to form complexes with other partners and stimulate their HRE-independent functions. Finally, HIF-1 is known to be downregulated upon prolonged exposure to hypoxia. A proposed mechanism for this phenomenon involves phosphorylation of the HIF-1α ODD domain by GSK-3 and consequent VHL-independent proteasomal degradation (Flugel et al., 2007; Mottet et al., 2003). Phosphorylation by CK1 and subsequent HIF-1 heterodimer disassembly could also serve as a mechanism promoting HIF-1 inactivation and termination of HRE-driven gene transcription upon prolonged hypoxia or another, as yet uncharacterized, stimulus.

Materials and Methods

Plasmid constructions

cDNAs encoding N-terminal fragments of HIF-1α were produced by PCR and cloned as *Bam*HI fragments into the pGEX-4T1 bacterial expression vector (Amersham), yielding pGEX-HIF-1α 1–71, 1–176, 72–176, 72–251, 72–229, 159–251, 159–229. HIF-1α point mutants were constructed using full-length HIF-1α cDNA cloned into pGEX-4T1 (Chachami et al., 2005) as template, the QuikChange II site-directed mutagenesis kit (Stratagene) and suitable primers (sequences available upon request). Wild-type and point-mutant forms of HIF-1α were subcloned as *Bam*HI fragments into the mammalian expression vector pEGFP-C1 (Clontech) and the yeast expression vector pBEVY-GU (Miller et al., 1998). Construction of pGEX-4T1-DM-HIF-1α (the double mutant of HIF-1α with S641A and S643A substitutions that is unable to be phosphorylated by MAPK) was previously described (Mylonis et al., 2006). pGEX-4T1-DM-HIF-1α-S247A and pGEX-4T1-DM-HIF-1α-S244A were constructed by replacing a *Hind*III-*Age*I digestion fragment of DM-HIF-1α with the *Hind*III-*Age*I digestion fragment of HIF-1αS247A or HIF-1αS244A, which contains the S247A or S244A mutation, respectively. DNA sequencing of the point mutants was performed by Cogenics. pCDNA3.1-CK1δ and pCDNA3.1-CK1δ-K38M (Knippschild et al., 1997; Wolff et al., 2005; Giamas et al., 2007) were kindly provided by Uwe Knippschild (Centre of Surgery, University of Ulm, Germany). pAC28-ARNT-bHLH-PAS, a bacterial expression plasmid encoding a fusion protein containing an N-terminal thioredoxin six-histidine (TrxH6) tag and the N-terminal part of human ARNT (amino acids 1–474) comprising the bHLH and the full PAS domain (Chapman-Smith et al., 2004), was kindly provided by Murray L. Whitelaw (Discipline of Biochemistry, University of Adelaide, Australia).

Cell culture, transfection and fluorescence microscopy

Human HeLa, HEK293T, HRG1 (kindly provided by Thomas Hellwig-Bürgel, Institute of Physiology, University of Luebeck, Germany) and Huh7 cells were cultured in DMEM containing 10% FCS and 100 U/ml penicillin-streptomycin (Gibco), supplemented with 250 μg/ml geneticin (Gibco) in the case of HRG1 cells, whereas hBSMCs (Clonetics) were cultured in DMEM F-12 containing 10% FCS and 100 U/ml penicillin-streptomycin. Transient transfections were performed as described previously (Mylonis et al., 2006), whereas co-transfection experiments were performed using a 1:2 ratio of HIF-1α and CK1δ cDNA, respectively. When required, cells were treated for 4–16 hours with 1 mM dimethyloxalyl glycine (DMOG; Alexis Biochemicals) or with CK1 inhibitors IC261 (2 μM; Sigma), CKI-7 (10 μM; Sigma) or PF670462 (0.5 μM; Tocris Bioscience). For hypoxic treatment, cells were exposed for 4–16 hours to 1% O_2, 95% N_2 and 5% CO_2 in an IN VIVO$_2$ 200 hypoxia workstation (Ruskinn Life Sciences). 24 hours post-transfection, cells were washed with PBS, lysed and luciferase activity was determined using the luciferase assay kit (Promega). HEK293T cells transiently transfected with GFP-tagged versions of HIF-1α were analyzed by fluorescence microscopy as previously described (Mylonis et al., 2008).

siRNA-mediated silencing of CK1δ

mRNA encoding CK1δ was targeted using Hs-CSNK1D5 HP validated siRNA (Qiagen) and AllStars siRNA (Qiagen) as negative control. Huh7 cells were incubated in serum-free DMEM for 4 hours with siRNA (10 nM) in the presence of Lipofectamine 2000 (Invitrogen), as described by the manufacturer. 44 hours post-transfection, cells were exposed to hypoxia and 4 hours later they were harvested, and cell lysates were prepared for luciferase assays or for western blot analysis.

RNA extraction and quantitative PCR

Total RNA from hBSMCs was isolated using the Trizol reagent (Invitrogen) and cDNA was synthesized with the High Capacity Reverse Transcription kit (Applied Biosystems). Real-time PCR was performed with SYBR GreenER qPCR SuperMix Universal (Invitrogen) in a Rotor gene 6000 instrument (Corbett). The mRNAs encoding VEGF, NDRG1 and PLOD2 were amplified using convenient primers (sequences available upon request). Each sample was assayed in duplicate for both target and internal control. Relative quantitative gene expression was calculated using the DDCT method and the relative expression software tool (REST), and presented as relative units.

Protein purification, phosphorylation, western blot, immunoprecipitation and immunofluorescence

GST-tagged mutant HIF-1α forms and their fragments were expressed in *Escherichia coli* and purified as previously reported for GST–HIF-1α (Chachami et al., 2005). His-tagged ARNT bHLH–PAS protein was expressed in *E. coli* and purified as previously reported for His–GFP–HIF-1α (Chachami et al., 2005). Phosphorylation reactions were carried out as previously reported (Mylonis et al., 2006) using, when required, 100 units of recombinant active CK1δ (New England Biolabs). CK1 inhibitor IC261 was used at 10 μM. Analysis by SDS-PAGE followed by western blotting, immunoprecipitation and immunofluorescence were carried out according to previously published procedures (Mylonis et al., 2008). ARNT was detected using a mouse anti-ARNT antibody (BD Transduction Laboratories), His-tagged ARNT bHLH–PAS was detected using a mouse anti-pentaHis-HRP-conjugated antibody (Qiagen), GST or GST-tagged proteins using a goat anti-GST antibody (Amersham) and CK1δ was detected using a rabbit anti-CK1δ antibody (Santa Cruz Biotechnology).

Yeast transformation and β-galactosidase assay

A yeast strain expressing human ARNT and carrying the reporter gene plasmid pHRE-*lacZ* was transformed with pBEVY-GU-derived plasmids expressing the wild-type or point-mutant forms of HIF-1α. Independent transformants were selected, cultured and assayed for β-galactosidase, as previously described (Braliou et al., 2006).

In vitro binding assays

Approximately 10 μg of purified His-tagged ARNT bHLH–PAS recombinant protein was bound on 25 μl Ni–NTA–agarose (Qiagen) beads and incubated with 10 μg purified GST, GST–HIF-1α or its mutant forms (S247A, S247D) for 4 hours at 4°C in 0.7 ml of 25 mM Tris-Cl pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100, 1 mM PMSF, 5 μM pepstatin, 1 μM aprotinin (all from Sigma) and 50 mM imidazole. At the end of the incubation, the beads were washed, and bound proteins were eluted by 1× SDS-PAGE loading buffer and analyzed by SDS-PAGE and western blotting. For the reverse experiment, GST alone, GST–HIF-1α or its mutant forms were bound on GSH–sepharose beads (Amersham), incubated with purified His-tagged ARNT bHLH–PAS protein (in buffer without imidazole) and analyzed using the same conditions as above. When required, bound HIF-1α and its mutant forms were treated with 1000 units of recombinant CK1δ and 1 mM ATP for 1 hour at 30°C before being allowed to bind to His-tagged ARNT bHLH–PAS.

Molecular modeling

Models of the HIF-1α PAS-B domain (residues 237–345 of human HIF1α) were generated by comparative modeling, based on the structure of the PAS-B domain from human HIF-2α (residues 239–47). The sequence identity of the two domains is 77%. The homology modeling programs Modeller (Marti-Renom et al., 2000) and Swiss Model (Arnold et al., 2006) were used for the construction of the models, AMBER 98 for energy minimization (Ponder and Case, 2003), and the PROCHECK (Laskowski et al., 1993) and Verify3D (Luthy et al., 1992) software for the validation of the structure. Templates used for the modeling were: the structure of isolated HIF-2α PAS-B (PDB code 1p97A) and the structures of its heterodimer with the C-terminal PAS domain of the ARNT subunit of the HIF-2 transcription factor in the presence and absence of small ligands (PDB ID codes 3F1N, 3F1O and 3F1P), in which Arg362 was back mutated to the original glutamic acid residue in this position. All templates and modeling algorithms used yielded essentially identical results for the structural model of HIF-1α PAS-B. The structures were visualized and molecular images were generated using PyMol (DeLano Scientific). The surface electrostatic potential was evaluated using the APBS module of PyMol (Baker et al., 2001).

Cell proliferation assay

Huh7 cells (0.5×10^3/well) were seeded into 96-well plates and incubated for 24 hours before being treated with 2 μM IC261 or with DMSO as solvent control for another 48 hours under normoxic or hypoxic conditions. To keep the effective IC261 concentration in the medium relatively constant, cells were washed once with PBS and fresh IC261- or DMSO-containing culture medium was added every 12 hours. At the end of the incubation period, the number of cells was colorimetrically determined with the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions. Values were normalized by control experiments in the absence of cells in 96-well plates supplied with culture medium and IC261 or DMSO alone. Cell proliferation was expressed as percentage of control from two independent experiments performed in triplicate.

Statistical analysis

Statistical differences between two groups of data were assessed using the unpaired *t*-test in the SigmaPlot version 9.0 software (Systat); $P<0.05$ was considered to be significant (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

We are grateful to Uwe Knippschild (Centre of Surgery, University of Ulm, Germany) and Murray L. Whitelaw (School of Molecular & Biomedical Science, University of Adelaide, Australia) for their generous gift of plasmids and Thomas Hellwig-Bürgel (Institute of Physiology, University of Luebeck, Germany) for kindly providing the HRG1 cell line. We thank all the other members of the Laboratory of Biochemistry, University of Thessaly, as well as two unknown reviewers for their useful comments and suggestions.

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http://jcs.biologists.org/cgi/content/full/123/17/2976/DC1

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