

The Transcriptional Activation Function of the HIF-like Factor Requires Phosphorylation at a Conserved Threonine*

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Katarina Gradin^{‡§}, Chikahisa Takasaki[‡], Yoshiaki Fujii-Kuriyama^{‡§}, and Kazuhiro Sogawa^{‡¶}

From the [‡]Department of Biomolecular Science, Graduate School of Life Science, Tohoku University, Sendai, 980-8578, Japan and the [§]Core Research for Evolutional Science and Technology, Japan Science and Technology, Tokyo 150-0002, Japan

The hypoxia-inducible factor (HIF)-1 α and the HIF-like factor (HLF) transcription factors are regulated at multiple levels including protein stabilization, nuclear import, and activation of transactivation, resulting in recruitment of coactivators such as the cAMP-response element-binding protein (CREB)-binding protein (CBP)/p300 and SRC-1. During low oxygen tension these proteins modulate a network of genes that are necessary for angiogenesis, erythropoiesis, and glycolysis. We report here that the C-terminal transactivation domain of HLF is phosphorylated on multiple sites and that phosphorylation on threonine 844 of HLF is necessary for the transcriptional activation function of the protein independently of the hypoxia condition. Importantly, using the mammalian two-hybrid system we demonstrate that a substitution of threonine 844 to an alanine decreased the enhanced transcriptional activation function mediated by CBP/p300.

Low oxygen tension (hypoxia) plays an important role in diseases such as cancer, cerebral and myocardial ischemia, and chronic lung and heart diseases (reviewed in Ref. 1). Oxygen tension is also an important factor for regulation of mammalian genes that are involved in angiogenesis, vasculogenesis, glucose metabolism, and apoptosis (for a review see Ref. 1). These oxygen-sensitive genes are regulated by the hypoxia-inducible factor (HIF)-1 α ,¹ (2, and reviewed in Ref. 3) or the homologue protein HIF-like factor (HLF) (4), which also is termed EPAS1, HIF-2 α , HRF, or MOP2 (reviewed in Ref. 5). The mechanism of activation of oxygen-regulated genes has been determined mainly by studies of HIF-1 α ; however, the important functional domains in HIF-1 α are highly conserved in HLF (4). Moreover, HIF-1 α appears to have a general role by conferring oxygen-dependent regulation to the transcription machinery in all cells, whereas HLF is cell type-restricted and plays a more

specialized role, according to analysis of knock-out mice of HIF-1 α and HLF (6–8). The N-terminal half of these proteins contains a basic helix-loop-helix domain (bHLH) and a PER-ARNT-SIM (PAS) homology domain. The basic domain is necessary for DNA-binding, and both the helix-loop-helix and the PER-ARNT-SIM homology domain are necessary for dimerization of HIF-1 α and HLF to their partner factor, the aryl hydrocarbon nuclear translocator (ARNT) protein. Under hypoxic conditions the heterodimer binds to specific consensus DNA sequences and up-regulates genes such as erythropoietin, vascular endothelial growth factor, and glycolytic enzymes (for a review see Ref. 1).

The stimulatory effect of hypoxia is caused by stabilization of the oxygen-sensitive HIF-1 α and HLF. At normoxia these two proteins are rapidly degraded through the ubiquitin-proteasome machinery that involves the von Hippel-Lindau tumor suppressor (pVHL) E3 ubiquitin ligase complex (9–11). pVHL interacts with the HIF proteins through a short peptide motif containing a hydroxylated proline (12–15). The prolyl hydroxylases that are necessary for post-translational modification are oxygen-dependent, resulting in lower levels of pVHL binding and thus stabilization of HIF-1 α (12–15). In the C-terminal half of both HIF-1 α and HLF two transactivation domains, the N-terminal transactivation domain and C-terminal transactivation domain (CAD) are located, and it has been shown that these transactivation domains interact with coactivators such as the histone acetyltransferase cAMP-response element-binding protein (CREB)-binding protein (CBP)/p300, SRC-1, and TIF-2 (16–18). It also has been reported that CAD is a direct target of redox regulation because the interaction of CBP/p300 and SRC-1 with HIF-1 α and HLF is increased by the redox factor 1 (REF-1) and by thioredoxin (17, 18). In addition, Lando *et al.* (19) recently have shown that hypoxic induction of CAD activity is regulated by hydroxylation of a conserved asparagine.

Recent studies have shown that HLF and HIF-1 α are phosphoproteins and that the proteins are phosphorylated in the hypoxic cell (20–22). The involvement of protein kinases has mainly been proposed using inhibitors for tyrosine and serine/threonine kinases that block the transactivation function of HLF and HIF-1 α (20, 23). More recently, it has been reported that the Ser/Thr kinase Akt regulates HIF-1 α stability (22), and some reports also suggest that mitogen-activated protein kinases phosphorylate HIF-1 α and increase the transcriptional activity of the protein (21). However, the sites for phosphorylation and the mechanism behind the enhanced transactivation function have not yet been elucidated.

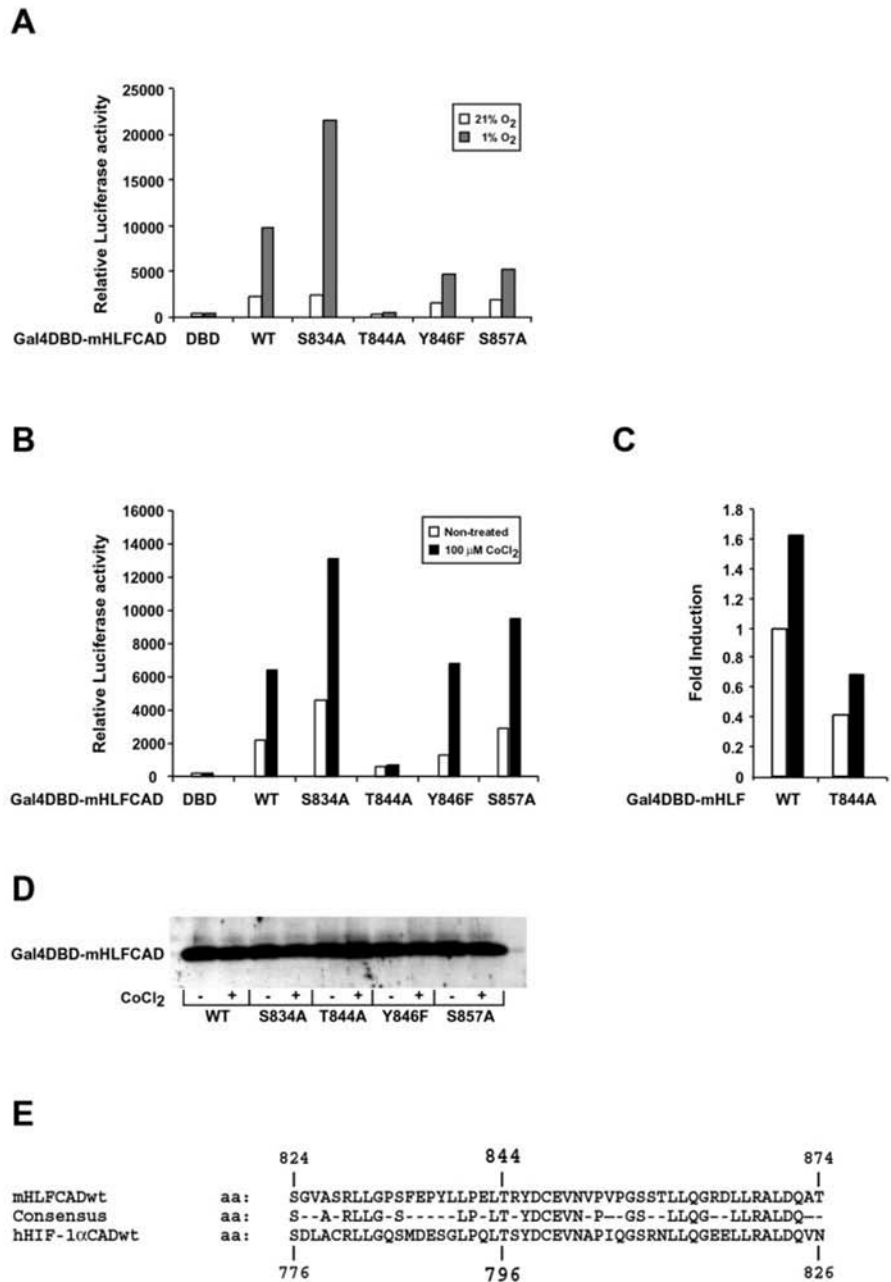
In this study we were interested to investigate the role of phosphorylation in modulating the transactivation function of HLF. To understand the role of phosphorylation of the C-terminal transactivation domain in a cellular context, we have

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¶ To whom correspondence should be addressed: Dept. of Biomolecular Science, Graduate School of Life Science, Tohoku University, Aobaku, Sendai, Miyagi, 980-8578, Japan. Tel.: 81-22-217-6591; Fax: 81-22-217-6594; E-mail: sogawa@mail.cc.tohoku.ac.jp.

¹ The abbreviations used are: HIF, hypoxia-inducible factor; HLF, HIF-like factor; ARNT, aryl hydrocarbon nuclear translocator; CAD, C-terminal transactivation domain; GST, glutathione-S-transferase; wt, wild type; HEK, human embryo kidney; Gal4DBD, Gal4-DNA binding domain; Gal4AD, Gal4-activation domain; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; m, mouse; h, human.

FIG. 1. Thr-844 mutated to an alanine abolished the transactivation capacity of HLF. HeLa cells were transiently cotransfected with 50 ng of Gal4DBD-HLFCAD fusion construct, wild-type or mutated (S834A, T844A, Y846F, and S859A), together with 0.5 μ g of Gal4-responsive reporter plasmid. Following transfection the cells were incubated either during normoxia (21% O₂) or hypoxia (1% O₂) (A) or in the presence or absence of 100 μ M CoCl₂ (B) for 18 h before harvesting. C, 1 μ g of Gal4DBD-mHLF chimeric construct, wild-type or T844A, were transiently transfected into HeLa cells together with a Gal4-luciferase reporter. The cells were subsequently treated with vehicle (H₂O) or 100 μ M CoCl₂ 18 h before harvesting. Luciferase activities were normalized for transfection efficiency by cotransfection of the β -galactosidase gene and by protein content. D, Western blot assay showing the expression levels of Gal4DBD fusion proteins. The cells were transfected with 3 μ g of the fusion plasmids. Whole cell extracts were prepared, and the proteins were separated on a 12% SDS-PAGE gel. The proteins were then transferred to a nitrocellulose membrane and visualized by an anti-Gal4DBD antibody. E, sequence comparison of mHLF and hHIF-1 α CAD.



analyzed putative phosphorylation sites that are conserved between HLF and HIF-1 α . To overcome the complex and interactive nature of post-translational modifications of HLF we have established these mutations in fusion proteins comprising the CAD of HLF and the heterologous DNA-binding domain of the Gal4 protein. By this approach we could analyze the CAD transactivation activity without the influence of other functional domains within HLF. In conclusion, we have shown that the transactivation capacity of both HLF and HIF-1 α depends on phosphorylation of the conserved residue Thr-844 and that this modification increases the affinity of the hypoxia-regulated proteins to the transcriptional coactivator CBP.

EXPERIMENTAL PROCEDURES

Cell Cultures and Transient Transfections—Human embryo kidney HEK 293T cells and human cervix carcinoma HeLa cells were routinely maintained in Dulbecco’s minimal essential medium and Eagle’s minimal essential medium, respectively, and supplemented with 10% fetal calf serum and 58 μ g/ml kanamycin. The cells were transiently transfected using the calcium phosphate DNA co-precipitation method as described previously (17). For reporter assay experiments, cells were

transiently transfected with expression plasmids (see legends to Figs. 1–4) together with a luciferase reporter gene carrying the upstream activation sequence (p(UAS)₃-luc) and pBos-LacZ. 6 h after transfection, the cells received fresh medium and were either exposed to normoxia (21% O₂) or hypoxia (1% O₂) or were treated with 100 μ M CoCl₂ or vehicle (H₂O) for 18 h before harvesting. The luciferase activity was determined using a luciferase detection kit (Promega) according to the manufacturer’s protocol. Luminescence was measured in a Victor multilabel plate reader (EG & G Wallac, Turku, Finland). The luciferase activity directed by various gene constructs was corrected for the β -galactosidase activity and protein content in the same samples, and each construct was analyzed as specified in the legends to Figs. 1, 2, and 4.

Plasmid Construction—Construction of pBos-Gal4DBD, pBos-Gal4DBD-HLFCADwt, p(UAS)₃-luc, and pBos-LacZ has been published previously (17). The different Gal4 DNA binding domain (Gal4DBD) HLFCAD-mutated plasmids were constructed by cloning an oligonucleotide containing the specific mutant that was spanning the region of HLFCAD between the *SalI* site and the *Sse8387I*. Oligonucleotides containing the different mutants with a *SalI* overhang in the 5’-end and a *Sse8387I* overhang in the 3’-end were then cloned into the *SalI*- and *Sse8387I*-digested pBos-Gal4DBD-HLFCADwt plasmid. A glutathione-S-transferase (GST) fusion construct, pBos-GST-HLFCADwt, was generated by inserting the blunt-ended *SalI*-*BalI* fragment of pBos-

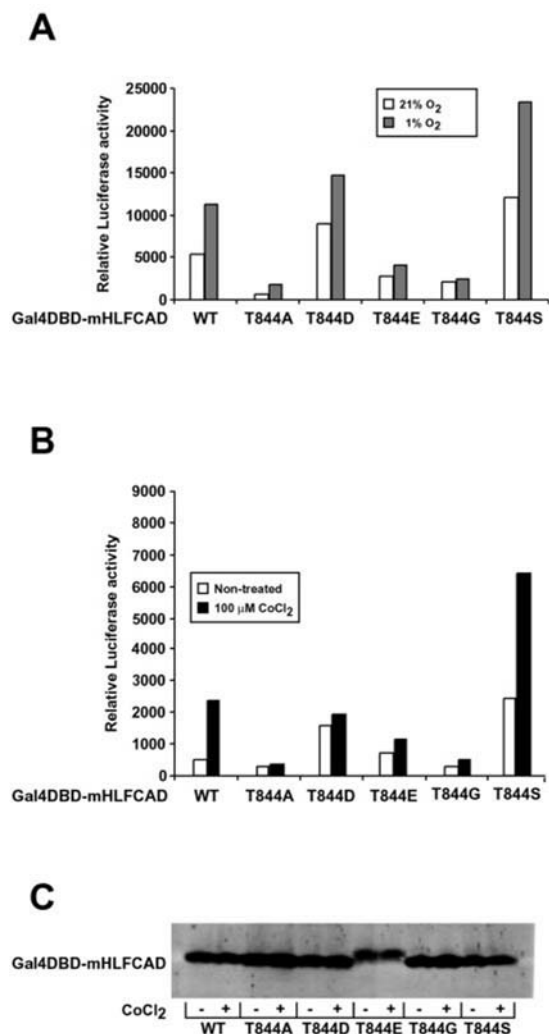


FIG. 2. Aspartic acid and serine restore the transactivation function of the alanine mutation on amino acid 844 in HLF. Thr-844 mutated to different amino acids (T844A, T844D, T844E, T844G, and T844S) were fused to the Gal4DBD and transiently transfected into HeLa cells. The cells were subsequently exposed to 21 or 1% oxygen (A) or 100 μ M CoCl₂ or vehicle (H₂O) (B) for 18 h before harvesting. The luciferase activities were normalized to the β -galactosidase activity and to the protein content. C, Western blot assay showing the expression levels of the Gal4DBD fusion proteins. The cells were transfected with 3 μ g of the fusion plasmids. Whole cell extracts were prepared, and the proteins were separated on a 12% SDS-PAGE gel. The proteins were then transferred to a nitrocellulose membrane and visualized by an anti-Gal4DBD antibody.

Gal4DBD-HLFCADwt into the *Sma*I site of pBosGST. pBos-Gal4DBD-HIF-1 α CADwt has been described previously (17), and pBos-Gal4DBD-HIF-1 α CADT796A and pBos-Gal4DBD-HIF-1 α CADT796D were constructed using the QuickChange site-directed mutagenesis kit (Stratagene) and pBos-Gal4DBD-HIF-1 α CADwt as template. pBos-Gal4AD CBP1–452 was constructed by inserting the blunt-ended *Hin*dIII fragment of pGAD424CBP1–452 (17) into the *Xba*I site of pBos vector, which had been treated with Klenow fragment. pBos-Gal4AD-CBP313–452 was constructed by deleting the *Eco*RV fragment from pBos-Gal4AD-CBP1–452. All DNA-constructions were validated by sequence analysis.

Western Blot—Because of low expression levels of the overexpressed proteins in HeLa cells, HEK 293T cells were used to compare the expression levels of various constructs. HEK 293T cells were transfected with wild-type and mutant constructs using the calcium phosphate DNA co-precipitation method. After 18 h the cells were harvested and lysed in radioimmune precipitation buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2, 2 mM EDTA) supplemented with 1 mM dithiothreitol and protease inhibitors (33 μ g/ml aprotinin, 0.5 mM phenylmethyl-

sulfonyl fluoride, 10 μ g/ml leupeptin, 5 μ g/ml pepstatin A). 30 μ g of the whole cell extract were resolved by SDS-PAGE, and the proteins were subsequently transferred to a nitrocellulose membrane (Amersham Biosciences). For Western blot analysis rabbit anti-Gal4DBD antibody (Upstate Biotechnology) diluted 1:1000 was used as first antibody, and goat anti-rabbit biotinylated IgG (Vector Laboratories) was used as secondary antibody. The membrane was then incubated with streptavidin-horseradish peroxidase complex (Vector Laboratories), and finally the ECL developing kit (Amersham Biosciences) was used to identify the relevant bands.

In Vivo Phosphorylation—HEK 293T cells cultured in a 6-cm dish were transfected with 3 μ g of either pBos-GST-HLFCADwt or pBos-GST-HLFCADT844A using the calcium phosphate DNA co-precipitation method. After 6 h the medium was changed, and the cells were cultured with or without 100 μ M CoCl₂ for 36 h. The cells were then cultured in phosphate-free medium for 1 h following an incubation with 100 μ Ci [³²P]orthophosphate (Amersham Biosciences) in phosphate-free medium for 4 h, and subsequently whole cell extracts were prepared in radioimmune precipitation buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2, 2 mM EDTA) supplemented with 1 mM dithiothreitol, 1 mM sodium orthovanadate, and protease inhibitors (33 μ g/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 5 μ g/ml pepstatin A). The whole cell extracts were incubated together with glutathione-Sepharose and bacterially expressed GST-HLFCAD that was used as a carrier protein for 2 h at 4 $^{\circ}$ C. The beads were then washed three times with lysis buffer, and the proteins were eluted by boiling for 5 min in SDS sample buffer. 10% of the eluted proteins were applied to a 12% SDS-PAGE gel, subsequently dried, and exposed to x-ray film, and quantitative analysis was performed using a STORM 850 PhosphorImager analyzer (Molecular Dynamics).

In Vitro Translation and GST Pull-down Assay—The *in vitro* translations were performed in the presence of [³⁵S]methionine (Amersham Biosciences) using T3 or T7 polymerase-coupled rabbit reticulocyte lysates (Promega) according to the manufacturer's instructions. The GST fusion protein CBP-(1–452) and GST alone were produced in and purified from *Escherichia coli* BL21 (Lys) strain as described (17). ³⁵S-labeled full-length HLF wt and HLF T844A were incubated with GST or GST-CBP-(1–452) adsorbed to glutathione-Sepharose beads (Amersham Biosciences) at 4 $^{\circ}$ C in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.2% Nonidet P-40, 1 mM dithiothreitol, 2 μ M EDTA, and protease inhibitors (33 μ g/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 5 μ g/ml pepstatin A). After an overnight incubation the beads were pelleted and washed three times with binding buffer. The proteins were then eluted by boiling for 5 min in 50 μ l of SDS sample buffer, and 20 μ l of the eluted proteins were applied to a 7% SDS-PAGE gel. The gel was subsequently dried and exposed to x-ray film, and quantitative analysis was performed using a STORM 850 PhosphorImager analyzer. Standardized amounts of purified GST fusion proteins, as determined by staining with Coomassie Blue, were used in the pull-down assay.

RESULTS

Mutation of Threonine 844 to an Alanine in the CAD of HLF Abolished the Transactivation Function of CAD—Two transactivation domains, N-terminal and C-terminal, have been identified previously in mouse HLF (17). These transactivation domains are localized between positions 450–571 and 824–876, respectively. Phosphorylation of specific amino acids in transactivation domains has been shown to be an important event for transcription mediated by many factors (p53, for example) (Ref. 24 and references therein). In order to delineate the role of phosphorylation for the function of the CAD of HLF we constructed fusion plasmids spanning the Gal4DBD and the CAD (containing different amino acid substitutions), providing a system in which transcriptional activity of this protein could be assayed without interference of the N-terminal transcription domain and/or degradation. We focused on four putative phosphorylation sites in the CAD that are conserved between HLF and HIF-1 α . The transcriptional activity of the mutants was compared with that of the wild-type protein following transient transfection of HeLa cells together with the luciferase reporter construct p(UAS)₃-luc and the β -galactosidase reporter construct pBos-LacZ, which was used as an internal

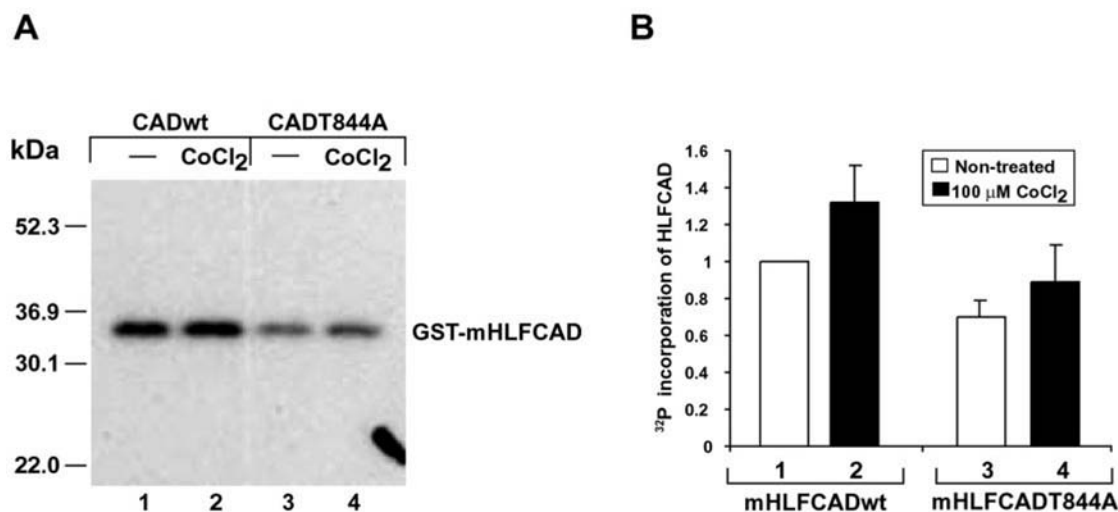


FIG. 3. *In vivo* phosphorylation of GST-HLFCAD. A, 293T cells were transfected with 3 μ g of either GST-HLFCADwt or GST-HLFCADT844A followed by a 36-h incubation with 100 μ M CoCl₂ or vehicle (H₂O). The cells were then labeled with 100 μ Ci [³²P]orthophosphate for 4 h in phosphate-free medium. Whole cell extracts were prepared, and the GST fusion proteins were immobilized on glutathione-Sepharose beads for 2 h and eluted by boiling in SDS sample buffer. 10% of the eluted material was analyzed by SDS-PAGE (12%) and visualized by autoradiography. B, the relative incorporation of ³²P into HLF CAD was quantified by phosphorimaging, and the extent of ³²P incorporation of HLF CADwt was defined as fold induction of non-treated HLF CADwt. The data represent three independent experiments; mean \pm S.E. is shown.

control. As a negative control pBos-Gal4DBD was used. In the presence of the latter protein very low levels of luciferase activity were observed regardless of whether the cells had been exposed to normoxia or hypoxia (Fig. 1A) or CoCl₂ (Fig. 1B). The Gal4DBD-HLF-CADwt protein showed a 50-fold increase in transcriptional activity at normoxia compared with Gal4DBD, which was further enhanced four times at hypoxia (Fig. 1A). The same pattern was observed when the cells were treated with CoCl₂ (Fig. 1B), which mimics the hypoxic condition, probably by inhibition of the proline and asparagine hydroxylases. When tyrosine 846 was mutated to a phenylalanine no differences in activation of HLF CAD was detected when compared with wild-type CAD. However, both of the mutations S834A and S857A produced a slight increase in the transactivation capacity. Interestingly, when the threonine at position 844 was substituted to an alanine the transactivation was totally abolished to almost the same levels as the Gal4DBD protein. To investigate whether the threonine was important also in the context of full-length HLF we constructed a Gal4DBD-HLF fusion protein. As shown in Fig. 1C the transcription activity was decreased to ~50% in the chimeric protein containing the T844A mutation. Hypoxia-dependent activation could still be detected, which probably is mediated by the N-terminal transactivation domain. These results indicate that threonine 844 is necessary for CAD-mediated transcriptional activation by HLF, and it encouraged us to study further the importance of Thr-844 in HLF CAD-mediated transcription.

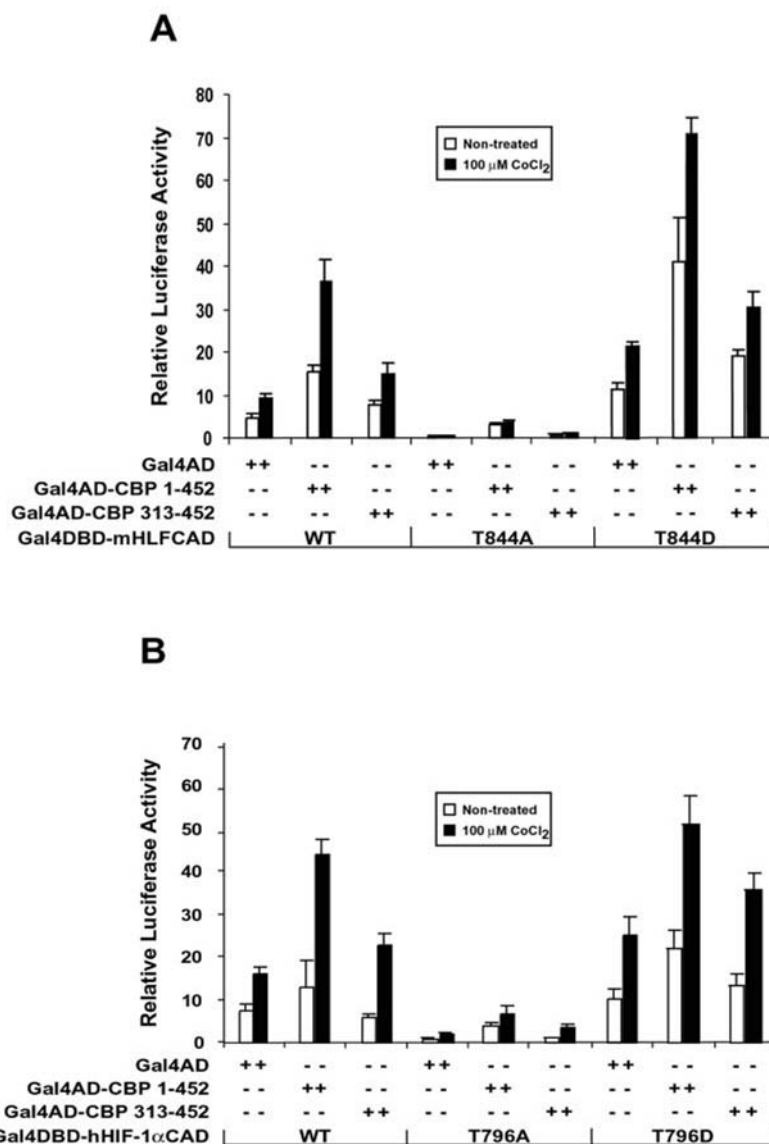
The Transactivation Function of HLF CAD Mutant T844A Is Restored by Aspartic Acid or Serine Substitutions—To investigate whether phosphorylation on the threonine is required for HLF CAD activity, we performed a more detailed mutation analysis of Thr-844. It is well known that both aspartic acid and glutamic acid can mimic a constitutively phosphorylated state of the protein. When we mutated Thr-844 to Asp constitutive activation of the luciferase reporter gene was observed, irrespective of normoxic or hypoxic conditions (Fig. 2, A and B). However, substitution of Thr-844 to glutamic acid or to glycine abrogated the transcription activation function of HLF CAD to almost the same level as T844A (Fig. 2, A and B). Many kinases that phosphorylate threonine are also able to phosphorylate a serine residue. To test whether a substitution of Thr-844 to serine affected HLF CAD activity we performed transient

transfection with a construct where the threonine was replaced by a serine. Interestingly, this mutated protein showed no or very low difference in inducibility by hypoxia and CoCl₂ as compared with the wild-type protein. These results indicate that the potential kinase that phosphorylates the amino acid at position 844 recognizes both a threonine and a serine residue. All fusion proteins were expressed at equal levels except for the glutamic acid-containing peptide, which showed a slower migrating band (Fig. 2C). This result may explain why the glutamic acid mutant did not show constitutive activity in analogy to the aspartic acid construct. Taken together, these results show that Thr-844 is a potential phosphorylation site in HLF CAD and that this residue is necessary for the function of HLF CAD.

Multiple Phosphorylation Sites in HLF CAD—Next we analyzed the *in vivo* phosphorylation status of HLF CAD. HEK 293T cells were transiently transfected with pBOS-GST-mHLFCADwt or pBOS-GST-mHLFCADT844A. After treatment with vehicle or 100 μ M CoCl₂ for 36 h the cells were labeled with [³²P]orthophosphate for 4 h in phosphate-free medium, and subsequently the GST-mHLFCAD proteins were purified with glutathione-Sepharose. As shown in Fig. 3A wild-type HLF CAD was labeled *in vivo* with orthophosphate. However, when the threonine at position 844 had been substituted to an alanine residue ³²P labeling was reduced by ~35% (Fig. 3B). These results tell us that the HLF CAD is phosphorylated at multiple sites and that one of the phosphorylated sites is Thr-844.

CBP/p300 Interaction with HLF CAD and HIF-1 α CAD Was Decreased in the Alanine-substituted Proteins—It has been shown previously that CBP/p300 interacts with the CAD motifs of both HLF and HIF-1 α and enhances transactivation of the proteins (17, 18). It was of interest, therefore, to determine whether mutation of Thr-844 affected the interaction between HLF and CBP/p300, and to investigate this interaction we utilized the mammalian two-hybrid assay. pBos-Gal4DBD-HLF CADwt or pBos-Gal4DBD-HIF-1 α CADwt were transfected into HeLa cells together with a fusion protein between the Gal4 activation domain (Gal4AD) and the N-terminal domain of CBP/p300 containing the CH1 moiety, which previously has been shown to mediate the interaction between HLF CAD and HIF-1 α CAD with CBP/p300 (17, 25). Both Gal4DBD-

FIG. 4. CBP/p300 interaction with HLFCAD and HIF-1 α CAD. *A*, mammalian two-hybrid assay was performed using in 50 ng of pBos-Gal4DBD-HLFCADwt, pBos-Gal4DBD-HLFCADT844A, or pBos-Gal4DBD-HLFCADT844D together with 150 ng of pBos-Gal4AD-CBP1-452 or pBos-Gal4AD-CBP313-452. *B*, 50 ng of pBos-Gal4AD-CBP1-452 or pBos-Gal4AD-CBP313-452. *B*, 50 ng of pBos-Gal4DBD-HIF-1 α CADwt, pBos-Gal4DBD-HIF-1 α CADT796A, or pBos-Gal4DBD-HIF-1 α CADT796D were co-transfected with 150 ng of pBos-Gal4AD-CBP1-452 or pBos-Gal4AD-CBP313-452. Following transfection the cells were treated with 100 μ M CoCl₂ or vehicle (H₂O) for 18 h before harvesting. Luciferase activities were normalized by the β -galactosidase activity and by protein content. The results represent two independent experiments performed in triplicate; mean \pm S.E. is shown.



HLFCADwt and Gal4DBD-HIF-1 α CADwt showed increased transcription activation in the presence of Gal4AD-CBP1-452 and CoCl₂ (Fig. 4, A and B). However, when the shorter fragment of CBP (Gal4AD-CBP313-452) was used very little effect was detected on the transactivation capacity of both HLF and HIF-1 α in comparison with cells that had been transfected with the parental Gal4AD construct (Fig. 4, A and B). These results indicate that not only the CH1 domain but also the most N-terminal part of CBP is necessary for efficient interaction with HLF and HIF-1 α . When Thr-844 in HLFCAD was mutated to an alanine the truncated chimeric CBP proteins had no effect or very little effect on the reporter gene activity. The same result was obtained when the corresponding threonine in HIF-1 α CAD, Thr-796, was mutated to an alanine (Fig. 4, A and B). However, when Thr-844 in HLFCAD and Thr-796 in HIF-1 α CAD were mutated to an aspartic acid the transactivation function of either protein was further enhanced in the presence of Gal4AD-CBP1-452 (Fig. 4, A and B). This effect was observed in two different cell lines upon exposure to hypoxia or CoCl₂ treatment, demonstrating that the result was not caused by the cell type or the culture condition.

To further investigate whether threonine 844 in HLFCAD was necessary for the interaction with CBP we used a GST pull-down assay. Recombinant GST-CBP-(1-452) or GST alone

was incubated with *in vitro*-translated ³⁵S-labeled full-length HLF or the HLF T844A mutant (Fig. 5A). A strong interaction could be detected when GST-CBP-(1-452) was incubated with wild-type HLF (Fig 5A, lane 3). However, when Thr-844 was substituted with an alanine the interaction was decreased ~70% (Fig. 5, A and B, compare lanes 3 and 6), indicating that Thr-844 is important for the interaction with CBP. In addition, no binding was observed when the labeled HLF proteins were incubated with GST alone (Fig. 5A, lanes 2 and 5). These results show that the specific threonine residue is necessary for CAD function not only in the context of HLF but also in the case of HIF-1 α . In addition, phosphorylation of the conserved threonine appears to be necessary for interaction of HLF- and HIF-1 α -CAD with CBP/p300.

DISCUSSION

HLF and HIF-1 α are regulated at multiple levels, including protein stabilization (9-11, 14, 15), nuclear translocation, and transactivation (10, 17-19). The most investigated mechanisms so far concern the stabilization of HIF-1 α and the transcriptional activation function of HLF and HIF-1 α . Phosphorylation and dephosphorylation are common mechanisms by which many transcription factors are regulated. Previous reports have shown that both HIF-1 α and HLF are phosphoryl-

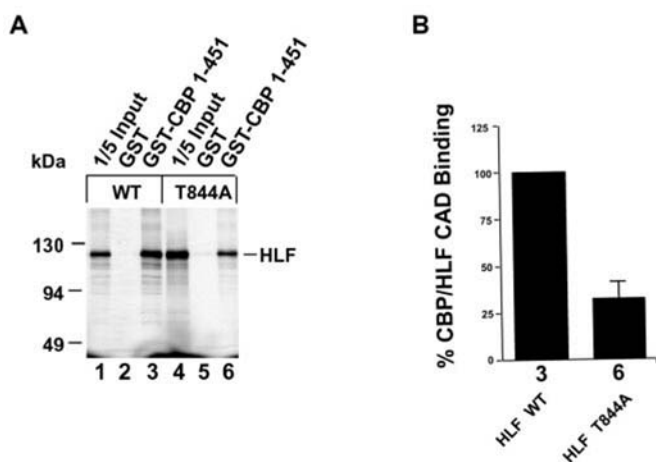


FIG. 5. **CBP/p300 interaction with full-length HLF.** A, *in vitro*-translated [35 S]methionine-labeled full-length mHLF wt (lanes 1–3) and mHLF T844A (lanes 4–6) were incubated overnight at 4 °C with recombinant GST-CBP-(1–452) or GST alone immobilized on glutathione-Sepharose. Bound proteins were eluted in SDS sample buffer and analyzed on a 7% SDS-PAGE gel. Lanes 1 and 4 show 20% of the amount of [35 S]methionine-labeled HLF proteins used in the binding buffer. Lanes 2 and 5 represent labeled HLF proteins incubated with GST alone, and lanes 3 and 6 represent labeled HLF proteins incubated with GST-CBP-(1–452). B, the relative incorporation of [35 S]methionine into HLF wt and HLF T844A was quantified by phosphorimaging, and the extent of [35 S]methionine incorporation in HLF wt was defined as 100%. The data represent three independent experiments; mean \pm S.E. is shown.

ated upon exposure to hypoxia and that the transcriptional activity is increased. However, the mechanism by which the transcriptional activity is enhanced is so far poorly understood (20, 21). We were therefore interested to investigate whether phosphorylation of the C-terminal transactivation domain influenced the transcription capacity of HLF. To do this, Gal4DBD fusion proteins were constructed containing wild-type HLF CAD or HLF CAD with different mutations at potential phosphorylation sites. We selected four different amino acids that are conserved between HLF and HIF-1 α . In transient transfections three of these amino acids, two serines and one tyrosine, showed no or only a slightly enhanced transactivation function compared with the wild-type protein. However, if the threonine at position 844 was changed to an alanine the transcription activity was completely abolished. These data suggest that the threonine is an important residue in transactivation by HLF. Moreover, the homologous threonine residue in HIF-1 α is equally important because HIF-1 α CAD containing an alanine instead of threonine 796 exhibits the same low functional activity that was produced by the HLF mutation.

At this stage the most important question was to verify the phosphorylation status of Thr-844. In the *in vivo* phosphorylation experiment T844A CAD was clearly phosphorylated. However, compared with the wild-type CAD the *in vivo* labeling was reduced \sim 35%. These results suggest that the HLF CAD is phosphorylated at multiple sites and that one of these sites is Thr-844. In addition, we have shown that a substitution of alanine to an aspartic acid fully restored the activity of HLF CAD. Taken together, these results demonstrate that the Thr-844 in HLF is phosphorylated and that a negative charge is required for the full functional activity of the HLF CAD. The data also indicate that there were no significant differences in the level of expression of the mutants compared with the wild-type CAD. In many reports it is shown that mitogen-activated protein kinases are activated by hypoxia (20, 21); however, no *in vitro* phosphorylation of HIF-1 α CAD could be detected by

p42/p44 mitogen-activated protein kinase.² The Thr-844 in HLF and the Thr-796 in HIF-1 α are consensus sites for casein kinase II (CKII); nevertheless, HLF CAD could not be phosphorylated *in vitro* by CKII (data not shown). However, this result may not exclude that the potential kinase for Thr-844 and Thr-796 is a CKII-like kinase.

It has been shown that CBP/p300 interacts with both HLF and HIF-1 α to mediate contact with the basal transcription machinery (17, 18). In addition, Kung *et al.* (25) have shown that the interaction of CBP/p300 with HIF-1 α CAD is necessary for hypoxia-inducible gene expression and for tumor growth. Using the mammalian two-hybrid system, we have shown that the enhanced transcription mediated by the CBP/p300 coactivator in wild-type CAD was very low if not totally abrogated in the context of an alanine substitution. Consistent with this observation, *in vitro* experiments demonstrate that Thr-844 in HLF CAD is important for interaction with CBP/p300. It has been observed previously that phosphorylation of the transactivation domain is necessary for CBP/p300 interaction by the tumor suppressor protein p53, where phosphorylation of Ser-15 increased the association of p53 with CBP/p300 in parallel with acetylation of the C-terminal domain, providing a link between phosphorylation and acetylation (26). Recently it has been shown that inhibition of hydroxylation of Asn-851 in HLF CAD under hypoxic conditions promotes the CAD region to interact with CBP/p300 (19); however, phosphorylation of Thr-844 in HLF CAD, regardless of the oxygen concentration, also seems to be necessary for the function of the transactivation domain. In summary, our data indicate that Thr-844 in HLF and Thr-796 in HIF-1 α are constitutively phosphorylated and that this phosphorylation is important for the transactivation function of these proteins.

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² F. Wang, K. Sogawa, and Y. Fujii-Kuriyama, unpublished observation.

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