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Molecular mechanisms of transcription activation by HLF and HIF1α in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300

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Hypoxia-inducible factor 1 α (HIF1α) and its related factor, HLF, activate expression of a group of genes such as erythropoietin in response to low oxygen. Transfection analysis using fusion genes of GAL4DBD with various fragments of the two factors delineated two transcription activation domains which are inducible in response to hypoxia and are localized in the C-terminal half. Their sequences are conserved between HLF and HIF1α. One is designated NAD (N-terminal activation domain), while the other is CAD (C-terminal activation domain). Immunoblot analysis revealed that NADs, which were rarely detectable at normoxia, became stabilized and accumulated at hypoxia, whereas CADs were constitutively expressed. In the mammalian two-hybrid system, CAD and NAD baits enhanced the luciferase expression from a reporter gene by co-transfection with CREB-binding protein (CBP) prey, whereas CAD, but not NAD, enhanced β-galactosidase expression in yeast by CBP co-expression, suggesting that NAD and CAD interact with CBP/p300 by a different mechanism. Co-transfection experiments revealed that expression of Ref-1 and thioredoxin further enhanced the luciferase activity expressed by CAD, but not by NAD. Amino acid replacement in the sequences of CADs revealed a specific cysteine to be essential for their hypoxia-inducible interaction with CBP. Nuclear translocation of thioredoxin from cytoplasm was observed upon reducing O_2 concentrations.

Keywords: oxygen sensing/protein stabilization/redox regulation/sulfhydryl modification/thioredoxin

Introduction

Oxygen is indispensable for maintenance of life in organisms from bacteria to mammals. Reduced oxygen concentrations (hypoxia) are sensed and cause a variety of adaptive reactions in these organisms (Bunn and Poyton, 1996). The hypoxic response can be invoked by treatment of the organisms with some transition metals (e.g. cobalt) or iron-chelating agents such as desferrioxamine. A heme-binding protein with oxygen sensor activity is found in bacteria (Gilles-Gonzalez *et al*., 1991) and has been proposed to exist in mammals (Goldberg *et al*., 1988), but the mammalian entity remains unidentified. At a molecular level, hypoxic conditions result in altered expressions of the genes for erythropoietin (Epo), vascular endothelial growth factor (VEGF), inducible nitric oxide synthase (iNOS), tyrosine hydroxylase, various glycolytic enzymes and others (Bunn and Poyton, 1996). Recent studies indicate that hypoxia-inducible factor 1 (HIF1) activates those genes through binding to an asymmetric E box-like element termed HRE, with a core sequence 5′-TACGTG-3′, in response to a low oxygen concentration (Semenza and Wang, 1992; Firth *et al*., 1994; Semenza *et al*., 1994; Levy *et al*., 1995; Melillo *et al*., 1995; Forsythe *et al*., 1996; Liu *et al*., 1996; for a review see Wenger and Gassmann, 1997). cDNA cloning has demonstrated that HIF1 is a heterodimer composed of HIF1α and HIF1β (Wang *et al*., 1995). The latter is a common factor already known as Ah receptor nuclear translocator (Arnt), which functions in association with Ah receptor (AhR) as a mediator of various biological and toxicological effects of dioxin and other xenobiotics (Hankinson, 1995; Sogawa and Fujii-Kuriyama, 1997), and has also been shown to play a critical role in angiogenesis of mouse embryos by the gene knockout technology (Maltepe *et al*., 1997). HIF1α is a novel member of the basic-helix–loop–helix (bHLH)-PAS [a conserved region among Per, Arnt and Sim (Nambu *et al*., 1991; Crews, 1998)] transcription factor family, which defines a subclass of the bHLH family (Wang *et al*., 1995) and participates in vascularization of mouse embryos (Carmeliet *et al*., 1998; Iyer *et al*., 1998; Ryan *et al*., 1998). More recently, a cDNA closely related to HIF1α has been isolated and its encoded protein product, termed HIF1α-like factor (HLF) (Ema *et al*., 1997) [also termed EPAS1 (Tian *et al*., 1997), HRF (Flamme *et al*., 1997), HIF2α (Wenger and Gassmann, 1997) or MOP2 (Hogenesch *et al*., 1997)] has been shown to function as an inducible transcription factor in association with Arnt in response to hypoxia. It is interesting to clarify how HLF and HIF1α sense low oxygen concentrations and transduce the transactivation signals to the basic transcriptional machinery.

Here, we have investigated mechanisms by which HLF and HIF1α activate the expression of target genes in response to hypoxia and describe the delineation of two independent minimal transcriptional activation domains, which are well conserved between HLF and HIF1α. One is designated NAD, for N-terminal transactivation domain [amino acids (aa) 450–571 of HLF and 481–603 of HIF1α], and the other is CAD, for C-terminal transactiv-

ation domain (aa 824–876 of HLF and 776–826 of HIF1α). These two activation domains have been identified recently with HIF1α by two other proups (Jiang *et al*., 1997; Pugh *et al*., 1997). The two domains behaved differentially in response to hypoxia. While NAD became stabilized under hypoxic conditions, CAD was modified by the thiol-redox regulators, Ref-1 and thioredoxin to interact with CREB-binding protein (CBP)/p300. When a conserved cysteine in the CAD was replaced by a serine, the ability of CAD to interact with CBP/p300 was abolished, resulting in loss of the transactivation activity in response to the $CoCl_2$ treatment mimicking the hypoxic conditions.

Results

Delineation of transcriptional activation domains of HLF and HIF1α

HLF or HIF1α activates transcription of reporter plasmids harboring the Epo HRE in a hypoxia-dependent manner (see review article by Wenger and Gassmann, 1997; Ema *et al*., 1997; Tian *et al*., 1997; Figure 1B, lanes 3, 4, 11, 12). Since bHLH-PAS transcription factors carry the transactivation domains in the C-terminal half (Franks *et al*., 1994; Jain *et al*., 1994; Whitelaw *et al*., 1994; Hirose *et al*., 1996; Li *et al*., 1996), we investigated effects of truncation of the C-terminal half of HLF, HIF1α and Arnt on the hypoxia-inducible gene expression (Figure 1A and B). Expression of one of the full-length HLF, HIF1α and Arnt with another of their C-terminal-truncated forms significantly decreased hypoxia-dependent transcriptional activation (Figure 1A and B, lanes 6, 8, 14, 16). Combined transfection of the C-terminal truncation mutant of HIF1α or HLF with that of Arnt reduced activation of the reporter gene expression by hypoxia to control levels that were observed following transfection with the empty expression vector, pBOS (Figure 1A and B, lanes 10 and 18). These results suggest that the C-terminal half of these factors is essential for hypoxia-inducible gene activation. To clarify the role of the C-terminus of HIF1α, HLF and Arnt in transactivation, we produced fusion genes by ligating the C-terminal portions of these factors to GAL DNA-binding domain (GALDBD) and co-transfected cells under normoxic or hypoxic conditions with these constructs together with a reporter plasmid carrying a tandem array of three GAL4-binding elements (UAS) upstream of the firefly luciferase gene (Figure 1C). Although GALDBD–Arnt and GALDBD–dSim constitutively activated the reporter plasmid as previously reported (Ema *et al*., 1996a) (data not shown), GALDBD–HIF1α and GALDBD–HLF markedly activated the luciferase expression in response to hypoxia and treatment with $CoCl_2$ (Figure 1C), indicating that the transcriptional activation domain which is responsive to hypoxia is located within the C-terminal half of HLF (this study) and HIF1α (Jiang *et al*., 1997; Pugh *et al*., 1997). To map the transcriptional activation domain in detail, we constructed numerous deletion mutants of GALDBD–HLF and GALDBD–HIF1α and transfected them into Hep3B and HeLa cells (Figure 1C). The results of these experiments were similar in both Hep3B and HeLa cells, and defined the two separate transcriptional activation domains in HLF and HIF1α molecules, NAD (aa 481–603 of HIF1α and 450–571 of HLF) and CAD (aa 776–826 of HIF1α and 824–874 of HLF).

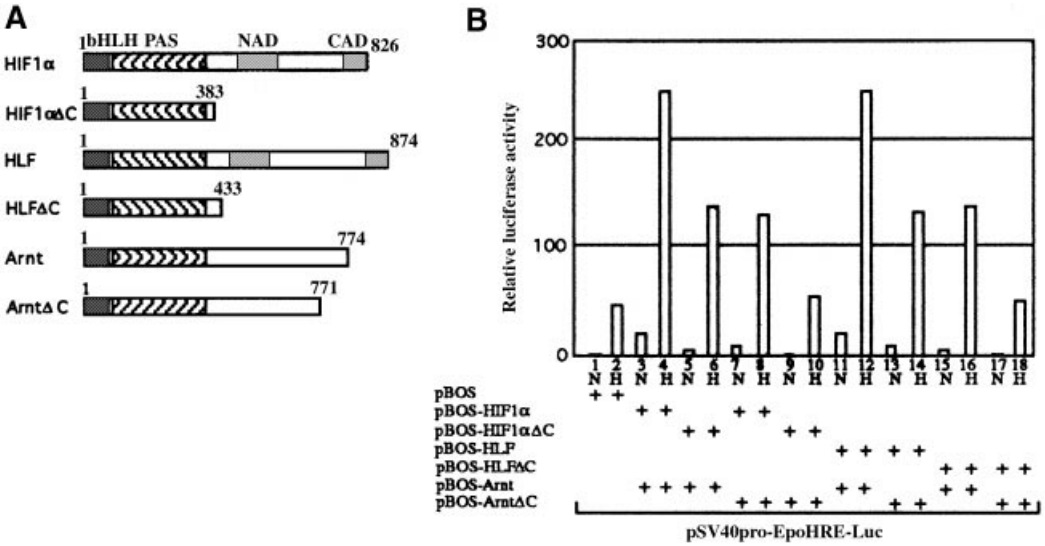
Expression level of HLF and HIF1α

To elucidate mechanisms of transcriptional activation by the two domains of HLF and HIF1α, we examined the expression levels of GALDBD–HLF and GALDBD–HIF1α fusion proteins in the absence or presence of $CoCl_2$ (Figure 2A). The expression levels of GALDBD, GALDBD–CAD–HIF1α and GALDBD–CAD–HLF under normal and $CoCl_2$-induced conditions were comparable, suggesting that changes in quality, but not in quantity of the CAD region (e.g. posttranslational modifications such as phosphorylation and redox modification) contribute to the hypoxic responsiveness. On the other hand, the expression levels of GALDBD–NAD–HIF1α and GALDBD–NAD–HLF were markedly enhanced by $CoCl_2$ treatment (>10- and ~3-fold, respectively). In normoxia, GALDBD–NAD–HIF1α protein was barely detectable, while GALDBD–NAD–HLF was expressed at a moderate level. Thus, induction ratio of HIF1α in response to $CoCl_2$ treatment was much greater than that of HLF, although maximal levels of HLF and HIF1α proteins were almost the same following $CoCl_2$ treatment. This tendency was also seen in full-length HLF and HIF1α protein (Figure 2C). Therefore, the transactivation by NADs of HLF and HIF1α in response to treatment with $CoCl_2$ can be explained at least in part by their increased protein levels. RT–PCR analysis of mRNA for HLF and HIF1α revealed essentially no change between normoxic and $CoCl_2$-induced conditions (Figure 2B), indicating that the increased protein levels of NADs of HLF and HIF1α are the result of posttranscriptional events. Recently, stabilization of HIF1α was suggested in response to hypoxia (Huang *et al*., 1996).

Role of CBP/p300 in hypoxia-inducible gene expression

It has been reported that CBP/p300 interacted with HIF1α and potentiated its transactivation activity (Arany *et al*., 1996). We were interested in investigating whether HLF also interacts with CBP/p300, and whether the transactivation domains of HLF and HIF1α are interactive interfaces with CBP/p300. Thus, we examined the effect of forced expression of CBP/p300 on hypoxia-inducible transactivation by the two minimal transcriptional activation domains of HLF and HIF1α in HeLa cells (Figure 3A). While it showed no significant effect on transcriptional activation by GALDBD (Figure 3A, lanes 1–4), overexpression of CBP/p300 markedly enhanced hypoxia-inducible transactivation by both NAD and CAD of HIF1α and HLF (Figure 3A, lanes 7–30), suggesting that CBP/p300 mediates hypoxia-inducible transcriptional activation by the two activation domains of HLF and HIF1α. p300 seemed to be a little more effective for enhancement of the transactivation of the two activation domains than CBP.

To investigate whether NAD and CAD interact with CBP, we used the yeast two-hybrid system. We constructed yeast expression plasmids encoding fusion proteins of GALDBD and various parts of HIF1α or HLF, and that of GAL activation domain (GALAD) and full-length CBP, and transfected the expression plasmids of GAD–CBP and one of the partner GALDBD fusion proteins into yeast for the liquid culture assay (Figure 3B). NADs of HLF and HIF1α themselves exhibited fairly strong transactivation activities in yeast, but the expressed activity



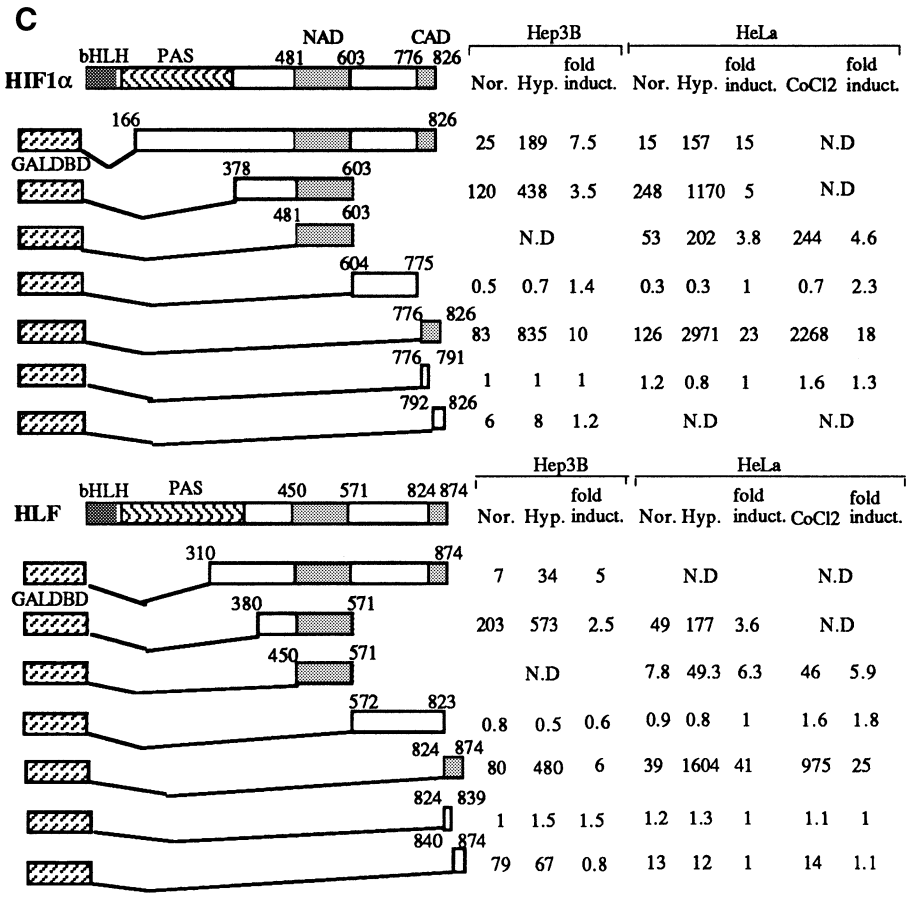


Fig. 1. Role of the C-terminal halves of HLF, HIF1α and Arnt in hypoxia-dependent transactivation. (**A**) Schematic representation of HIF1α, HLF and Arnt expression plasmids. Characteristic domains are indicated around the corresponding regions. (**B**) Effects of C-terminal truncation of HLF, HIF1α and Arnt on the hypoxia-inducible transcriptional activation. Combinations of effector and reporter plasmids (1 μg/plate) used for transfection by the calcium phosphate precipitate method are indicated below the figure. Cells were grown under normoxic (N, 21% O_2) and hypoxic (H, 1% O_2) conditions for 40 h and cell extracts were prepared by freezing and thawing method and subjected to luciferase assay. (**C**) Delineation of the transcriptional activation domain in response to hypoxia in HeLa and Hep3B cells and to $CoCl_2$ treatment in HeLa cells. GALDBD–HLF and GALDBD–HIF1α chimera plasmids (1 μg/plate) shown on the left side were transfected into Hep3B and HeLa cells with the reporter plasmid and then grown for 40 h under hypoxic conditions or in the presence of 200 μM $CoCl_2$. Numbers above schematic structures of the expression plasmids are the amino acid positions as defined previously (Ema *et al.*, 1997). These fusion constructs were produced by using appropriate restiriction cleavage sites or the RT–PCR method using appropriate primers and then ligating these fragment with T4 ligase. The sequences of the constructs were confirmed by squencing. Thin solid lines indicate the deleted sequences between the fused domains.

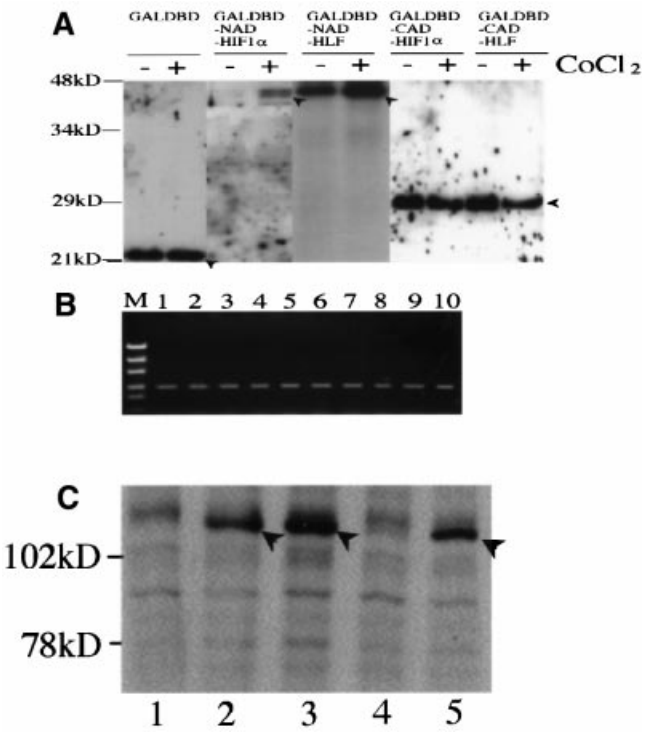


Fig. 2. Expression levels of HLF and HIF1α under normoxic and hypoxic conditions. (**A**) Immunoblot analysis of GALDBD, GALDBD–HLF and GALDBD–HIF1α fusion proteins extracted from the cells grown under normoxic conditions and in $CoCl_2$ solution. GALDBD–HLF or GALDBD–HIF1α chimera plasmid was transfected into HeLa cells and the cells were grown for 40 h under normoxic conditions. Then, whole-cell extracts were prepared from cells under normoxic conditions or treated with 200 μM $CoCl_2$ for 4 h. Immunoblotting was performed as described in the Materials and methods. Arrowheads indicate the bands for various GALDBD fusion proteins. (**B**) RT–PCR analysis of HLF and HIF1α mRNAs. A pair of primers for GALDBD region were used to amplify cDNAs. M, marker DNA. (**C**) Immunoblot analysis of full-length HLF and HIF1α in HeLa cells grown in normoxia and treated with $CoCl_2$. Expression plasmids of FLAG-epitope tagged full-length HLF and HIF1α are introduced into HeLa cells by the calcium phosphate precipitation method and the cells were grown in the presence of [^{35}S]methionine as described in Materials and methods under normoxic and $CoCl_2$-treated conditions. The cell extracts were prepared and were immunoprecipitated by anti-FLAG antibody for analysis of gel electrophoresis. Lane 1, empty vector; 2, HLF in normoxia; 3, HLF after 4 h of $CoCl_2$ treatment; 4, HIF1α in normoxia; 5, HIF1α after 4 h of $CoCl_2$ treatment. Arrowheads indicate the expressed protein.

was not further enhanced by the addition of the CBP plasmid. This result indicates that NAD was not able to interact with CBP in yeast for unknown reasons or some factor which mediates the interaction between NAD and CBP in HeLa cells may be missing in this cell. On the other hand, while CAD of HIF1α and HLF by themselves displayed no transactivating activity, addition of the CBP plasmid stimulated marked expression of β-galactosidase, indicating that CADs of the two transcription factors interacted with CBP in yeast.

To delineate the interaction domain of CBP with the CADs, we constructed expression plasmids encoding fusion proteins of CAD and fragments of CBP for the filter assay in the yeast two hybrid system. As shown in Figure 3C, the N-terminal region (aa 1–452) of CBP was identified as interacting with CAD.

Role of Ref-1/thioredoxin system in posttranslational activation of HLF and HIF1α

Ref-1 and thioredoxin, which function as redox-state regulatory factors, are known to activate hypoxia-inducible gene expression by HIF1α (Huang *et al*., 1996). Therefore, we examined the effect of Ref-1 and thioredoxin on the transcriptional activation by NAD and CAD (Figure 4). Expression of Ref-1 or thioredoxin enhanced the transactivation by CAD, but not by NAD in a hypoxia-dependent manner. Thioredoxin has five cysteine residues, two of which are known to be essential for redox reactions (Hirota *et al*., 1997). A mutant that has amino acid substitutions unable to catalyze the redox reaction revealed no effect on the transcriptional activation by CAD (Figure 4B, lanes 19, 20, 39, 40), suggesting that cellular redox signal transduction is responsible for hypoxia-inducible transcriptional activation by CAD. Interestingly, a single residue of cysteine is conserved in the CAD sequence of 49 amino acids between HLF and HIF1α (YDCEV) (Figure 4A). Replacement of this cysteine with a serine residue completely abolished the hypoxia-inducible transcriptional activation by CAD (Figure 4B). Since replacement of Cys with Ser did not alter the levels of expression of these proteins, irrespective of the presence or absence of $CoCl_2$ (Figure 4C), these results indicated that the cysteine residue was essential for the hypoxia-inducible transcription and was modified by Ref-1 and thioredoxin, probably to form a sulfhydryl group important for the transcriptional activation. The importance of this cysteine residue for the transcriptional activation was verified by the experiments using entire molecules of HIF1α and HLF with a single amino acid replacement. A replacement of cysteine with serine in the two molecules markedly reduced the transcription enhancing activity (Figure 4D). This remarkable reduction in the transcription activity of the Cys to Ser mutants of HIF1α and HLF was shown to be due probably to loss of interaction between the CADs and CBP as shown by the mammalian two-hybrid system. The fusion proteins of GALAD and N-terminal part of CBP interacted with the GALDBD–CAD, resulting in the enhanced expression of luciferase activity, but not with the mutated GALDBD–CAD having the serine substitution (Figure 5). These results indicate that the specific sulfhydryl group of the cysteine residue is essential for interaction with CBP.

In vitro interaction between CAD and CBP

To further investigate the interaction between HLF or HIF1α and CBP, we prepared the glutathione *S*-transferase (GST)-tagged CAD of HLF and HIF1α in bacteria and performed the GST pull-down assay (Figure 6). GST–CAD–HIF1α, but not GST–CAD–HIF1α with the serine substitution are able to bind to an *in vitro* synthesized CBP. Treatment of GST–CAD–HIF1α with *N*-ethylmaleimide, a sulfhydryl modifying reagent, also abolished its interaction with CBP, suggesting that free SH group of the cysteine residue is essential for the interaction with CBP. Interaction between CBP and GST–CAD–HLF was not detectable by this method, probably because CAD of HLF has lower affinity for CBP than that of HIF1α, as suggested by the transfection assay (Figures 3 and 5).

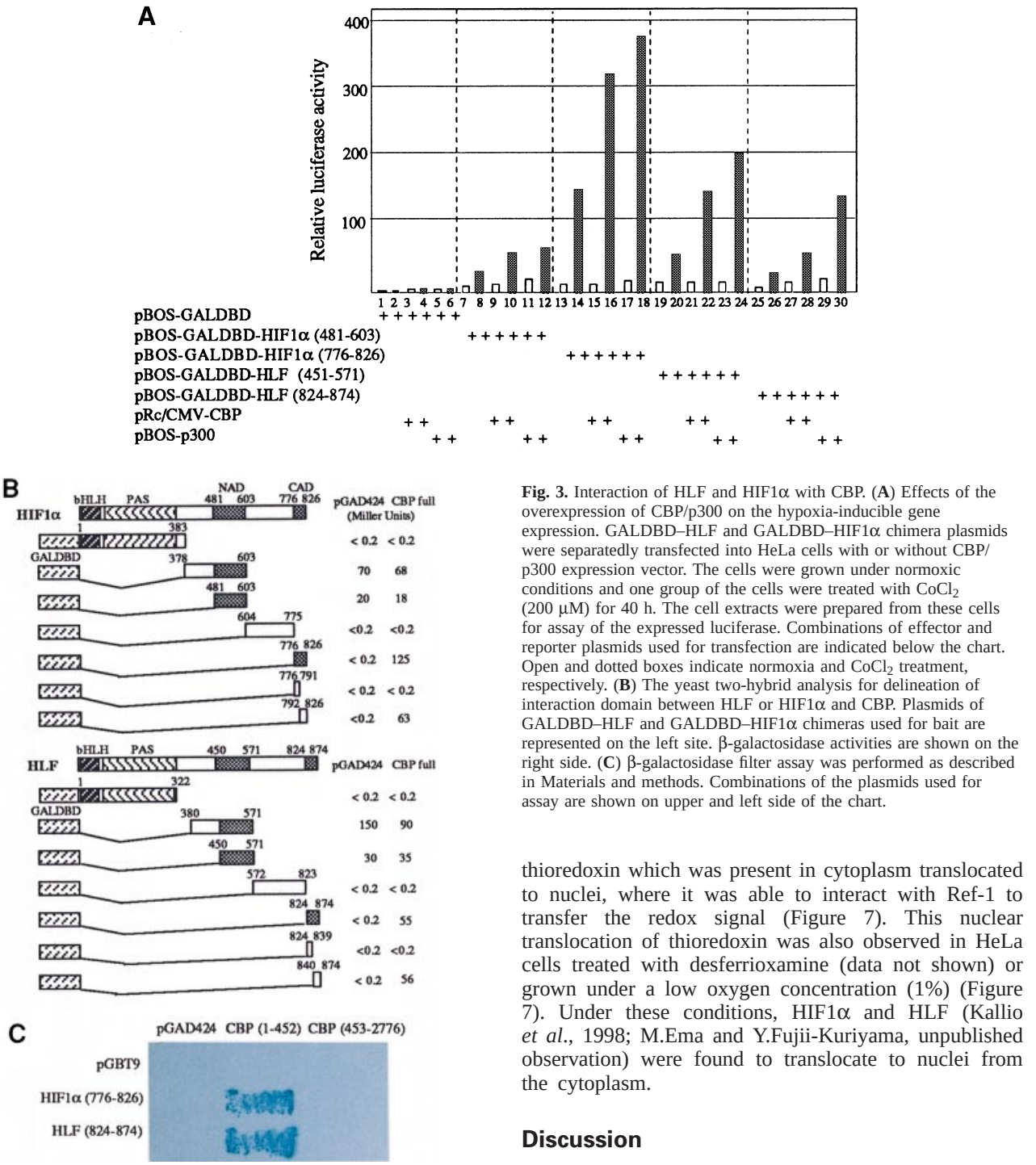


Fig. 3. Interaction of HLF and HIF1α with CBP. (**A**) Effects of the overexpression of CBP/p300 on the hypoxia-inducible gene expression. GALDBD–HLF and GALDBD–HIF1α chimera plasmids were separatedly transfected into HeLa cells with or without CBP/p300 expression vector. The cells were grown under normoxic conditions and one group of the cells were treated with $CoCl_2$ (200 μM) for 40 h. The cell extracts were prepared from these cells for assay of the expressed luciferase. Combinations of effector and reporter plasmids used for transfection are indicated below the chart. Open and dotted boxes indicate normoxia and $CoCl_2$ treatment, respectively. (**B**) The yeast two-hybrid analysis for delineation of interaction domain between HLF or HIF1α and CBP. Plasmids of GALDBD–HLF and GALDBD–HIF1α chimeras used for bait are represented on the left site. β-galactosidase activities are shown on the right side. (**C**) β-galactosidase filter assay was performed as described in Materials and methods. Combinations of the plasmids used for assay are shown on upper and left side of the chart.

Nuclear translocation of thioredoxin

Thioredoxin is known to exist in cytoplasm under normoxic conditions (Hirota *et al*., 1997). On the other hand, HLF and HIF1α translocate from cytoplasm to nuclei upon treatment with $CoCl_2$ (Kallio *et al*., 1998; M.Ema and Y.Fujii-Kuriyama, unpublished result) and Ref-1 is exclusively localized in nuclei (Xanthoudakis and Curran, 1992). We were interested to investigate where thioredoxin interacts with Ref-1 to transfer the thiol-redox signal finally to the cysteine of CADs of HLF and HIF1α. Indirect immunohistochemistry clearly demonstrated that upon $CoCl_2$ treatment of HeLa cells, thioredoxin which was present in cytoplasm translocated to nuclei, where it was able to interact with Ref-1 to transfer the redox signal (Figure 7). This nuclear translocation of thioredoxin was also observed in HeLa cells treated with desferrioxamine (data not shown) or grown under a low oxygen concentration (1%) (Figure 7). Under these conditions, HIF1α and HLF (Kallio *et al*., 1998; M.Ema and Y.Fujii-Kuriyama, unpublished observation) were found to translocate to nuclei from the cytoplasm.

Discussion

We have described the delineation of the two minimal transcriptional activation domains in the C-terminal half of HLF and HIF1α. One is designated NAD (aa 450–571 of HLF and 481–603 of HIF1α) and the other is CAD (aa 824–876 of HLF and 776–826 of HIF1α). The two domains were found to fall into the two highly conserved regions between HLF and HIF1α (Ema *et al*., 1997). Concerning HIF1α, the two separate transcriptional activation domains are in accordance with the previous reports on the activation domains of HIF1α defined by Pugh *et al*. (1997) and Jiang *et al*. (1997) but at variance with that of Li *et al*. (1996). Although these two domains activated the gene expression in response to hypoxia, their mechanisms of gene activation were revealed to be different.

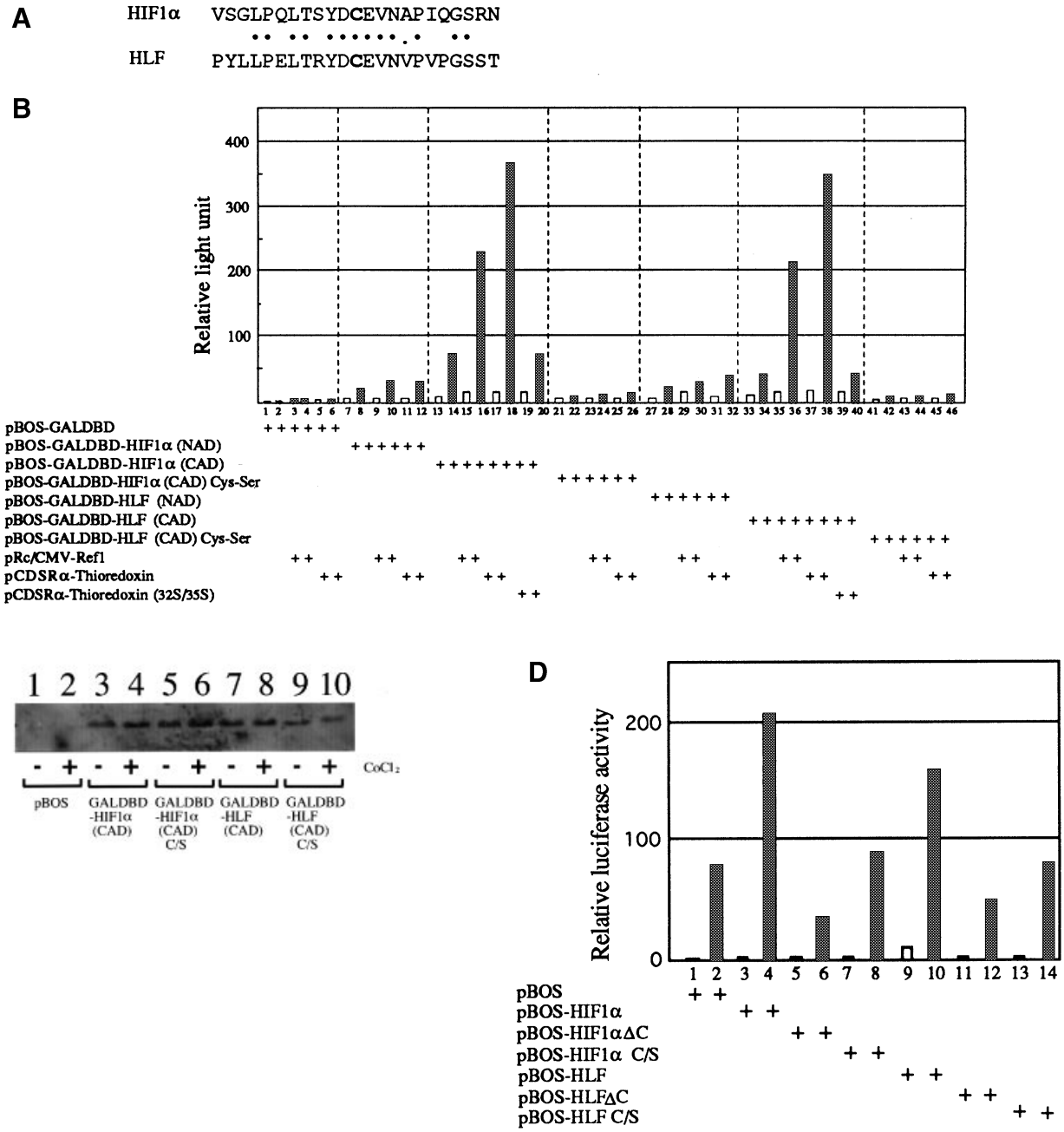


Fig. 4. Regulation of transcriptional activation by Ref-1 and thioredoxin. (**A**) Amino acid sequence around the conserved cysteine in CAD of HLF and HIF1α. Conserved cysteine residues are given by bold letters. (**B**) Effects of overexpression of Ref-1 and thioredoxin on the transcription activation by NAD, CAD and their Cys to Ser substituted forms on the hypoxia-inducible transcriptional activation. Various combinations of the expression plasmids (1 μg/plate) as indicated below the figure were transfected into HeLa cells together with the reporter plasmid, pG3E-luciferase (2 μg/plate). The transfected cells were grown for 40 h at 37°C in the presence or absence of $CoCl_2$ (200 μM). Open and dotted boxes indicate the absence and presence of $CoCl_2$, respectively. (**C**) Expression of GALDBD CADs of HIF1α and HLF and their Cys to Ser mutants in HeLa cells. The HeLa cells transfected with the expression plasmids of the fusion genes were treated as described in the legend to Figure 2 and were immunoblotted as described in Materials and methods.

Immunoblot analysis suggested that the protein levels of NAD of HLF as well as HIF1α were remarkably increased in response to hypoxia, while CAD protein levels of HLF and HIF1α remained more or less constant (Figure 2). Since RT–PCR analysis indicated that the mRNA levels of the fusion proteins containing NAD were not changed by hypoxia, the increased amounts of NAD proteins are considered to be due to posttranscriptional events. HIF1α protein in cells has already been reported to be rapidly degraded under normoxic conditions and became stabilized in hypoxia, resulting in increased amounts of HIF1α protein (Huang *et al.*, 1996; Kallio *et al.*, 1997). It has been reported that the DNA-binding activity of HIF1 to the HRE sequence as revealed by the gel mobility-shift assay was enhanced by inhibiting the ubiquitin–proteasome system (Salceda and Caro, 1997). Taken together, these results indicated that quantitative regulation of HLF and HIF1α in response to hypoxia is reflected in that of NAD and suggested that the NAD is a target of the ubiquitin–proteasome pathway in normoxia. An

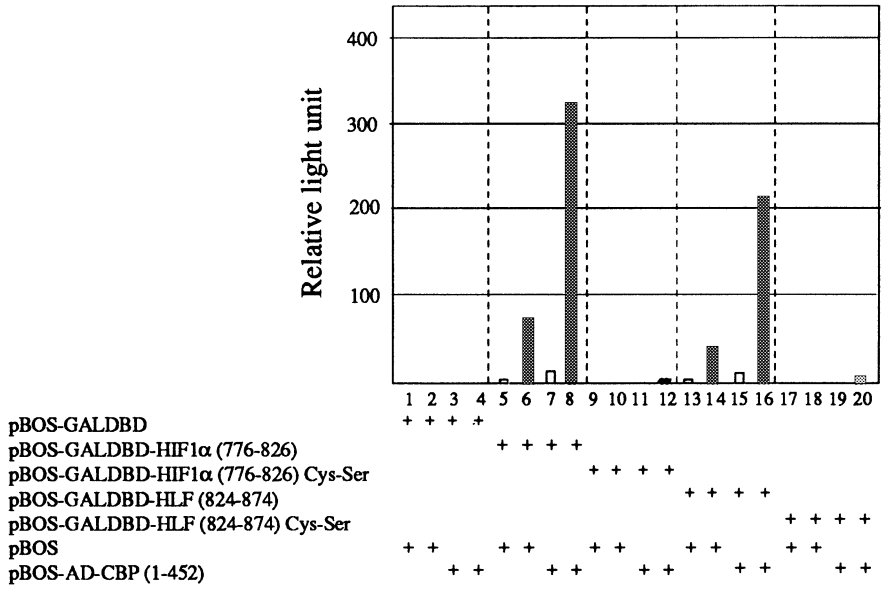


Fig. 5. Mammalian two-hybrid assay using various bait plasmids and GALAD/CBP (1–452) as prey. Co-transfection of each (1 μg/plate) of the various bait plasmids and the CBP prey plasmid (1 μg/plate) into HeLa cells was carried out together with the reporter gene, pG3E-luciferase (2 μg/plate). The transfected cells were grown in the presence or absence of $CoCl_2$. The expressed luciferase activity in the presence and absence of $CoCl_2$ are indicated by dotted and open boxes, respectively.

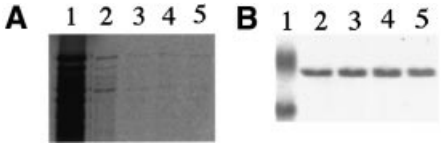


Fig. 6. Effects of a sulfhydryl reagent, *N*-ethylmaleimide (NEM) on the interaction between CBP and CAD of HIF1α. (**A**) GST pull-down assay with [^{35}S]methionine-labeled CBP. GST–CAD and GST–CAD (C/S) of HIF1α were bacterially synthesized and purified on a glutathione–Sepharose column. CBP was synthesized in the reticulocyte lysate system in the presence of [^{35}S]methionine and an aliquot of the reaction mixture was incubated with GST–CAD or GST–CAD (C/S) (2 μg) at 37°C for 20 min. The reaction mixtures were treated with glutathione beads. After the beads were washed with NETN, the adsorbed proteins were eluted from the beads by the Laemmli buffer and the eluates were analyzed by SDS–PAGE. GST–CAD–HIF1α was treated with NEM (50 mM) in NETN at 37°C for 20 min and then purified for GST pull-down assay. Lane 1, input protein; 2, GST–CAD–HIF1α; 3, GST–CAD–HIF1α (C/S); 4, NEM-treated GST–CAD–HIF1α; 5, NEM-treated GST–HIF1α (C/S). (**B**) Coomassie Brilliant Blue staining of a SDS–PAGE gel. Lane 1, molecular weight marker proteins; carbonic anhydrase (34 kDa) and soybean trypsin inhibitor (28 kDa). 2, GST–CAD–HIF1α. 3, GST–CAD–HIF1α (C/S). 4, NEM-treated GST–CAD–HIF1α. 5, NEM-treated GST–HIF1α (C/S).

oxygen-dependent degradation domain (ODD) which overlaps the NAD has been reported recently (Huang *et al.*, 1998).

On the other hand, the constant protein levels of CADs of HLF and HIF1α, irrespective of the absence or presence of $CoCl_2$, suggest that the hypoxic activation of the CAD activity of HLF and HIF1α is the result of qualitative regulation.

Co-transfection assays using expression plasmids of Ref-1 and thioredoxin showed that forced expression of Ref-1 and thioredoxin markedly enhanced the transactivation of the reporter gene by CAD but not by NAD, in a hypoxia-responsive manner. Interestingly, thioredoxin that exists in the cytoplasm under normoxic conditions was found to translocate to the nucleus where it is able to interact with Ref-1 to transfer the redox signal. A sulfhydryl-transfer defective mutant of thioredoxin revealed essentially no effect on transcriptional activation under hypoxic conditions, suggesting that cellular thiol-redox regulation is important for the hypoxia-inducible transcriptional activation by CADs of HLF and HIF1α.

Interestingly, the CADs of the two transcription factors contain a single conserved cysteine residue. Substitution of this cysteine with a serine residue completely abolished the transactivation activities of the two CADs, indicating that the conserved cysteine is essential for transactivation by CAD and is probably reduced by Ref-1 in response to hypoxia. This is in apparent contrast with the mechanism of redox regulation of the AP-1 transcription factor. The DNA-binding activity of Jun and Fos is strengthened by reduction of a conserved cysteine residue by Ref-1 as a redox regulator (Abate *et al.*, 1990; Xanthoudakis and Curran, 1992; Xanthoudakis *et al.*, 1992). Replacement of this cysteine residue with a serine, however, converts Jun and Fos into their constitutively active forms (Abate *et al.*, 1990). Importance of the thiol group of the cysteine in HLF and HIF1α was substantiated by interaction with CBP/p300. In the mammalian two-hybrid system, the Cys to Ser mutation in the CADs abolished the interaction between CADs and CBP, resulting in loss of transactivation activity of CADs. After submission of this paper, however, it has been reported very recently that a mutation of Ala at this Cys residue of HIF1α produced a modest reduction in activity, but appeared not to ablate inducibility (O'Rourke *et al.*, 1999). Although there are differences in mutated amino acids, Ala and Ser and in the cells used for transfection experiments between their experiment and ours, the apparent discrepancy cannot be understood clearly, and further work is needed to clarify the situation.

CBP/p300 is known to function as a general co-activator of various transcription factors (Kamei *et al.*, 1996) and was also found to mediate transcriptional activation by HIF1α (Arany *et al.*, 1996) and HLF (this study). Although

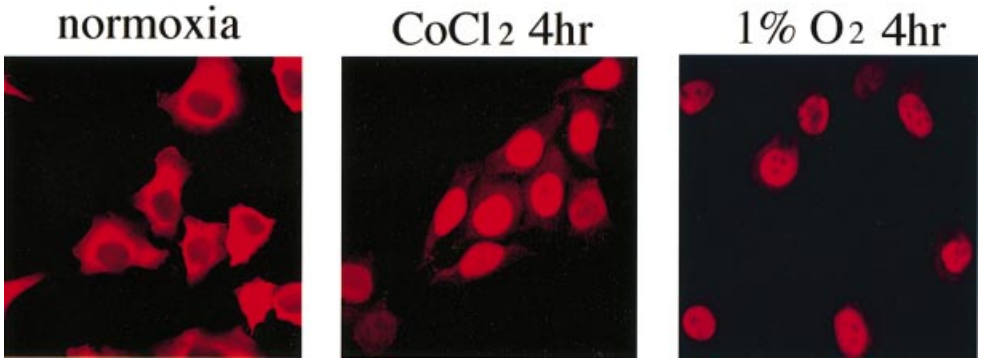


Fig. 7. Effects of hypoxic and $CoCl_2$ treatment on subcellular localization of thioredoxin. HeLa cells grown under normoxic, $CoCl_2$-treated (200 μM) and hypoxic (1% O_2) conditions for 4 h were subjected to indirect immunohistochemistry using an anti-thioredoxin antibody and observed with a confocal microscope.

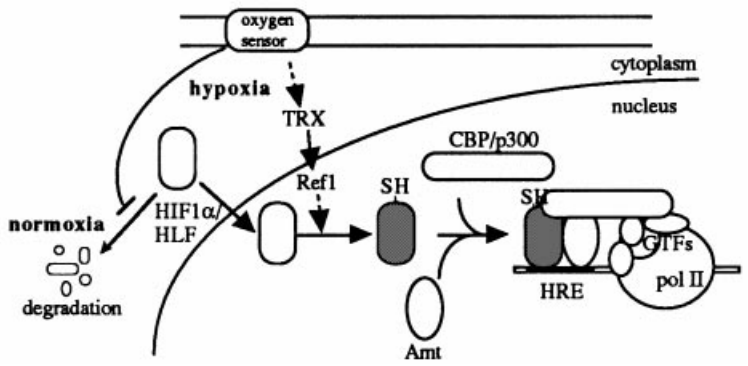


Fig. 8. Summary of molecular mechanisms of hypoxia-inducible transcriptional activation by HLF and HIF1α. Membrane-bound oxygen sensor is hypothetical. CBP is known to interact with the very C-terminal activation domain of Arnt (Kobayashi *et al*., 1997). GTFs, general transcription factors.

the two activation domains seem to interact with CBP/p300 as demonstrated by the mammalian two-hybrid system, the interaction of the two transactivation domains, NAD and CAD, with CBP/p300 is considered to be different as revealed by the yeast two-hybrid system. In this system, NAD showed intrinsic transactivation activity and this activity was not further enhanced by co-transfection with the CBP expression plasmid, while marked CAD transactivation activity was observed only in the presence of CBP. Taken together, these results suggest that CAD interacts directly with CBP, whereas interaction of NAD with CBP is mediated by a factor which appears to be lacking in yeast. A similar situation has been reported with regard to the interaction between Rb and HDAC (Brehm *et al*., 1998).

In conclusion, inducible activation of HLF and HIF1α proteins in response to hypoxia is produced by at least two mechanistic ways as summarized in Figure 8. One involves hypoxia-dependent stabilization of HLF and HIF1α proteins through their NAD sequence (this study; Jiang *et al*., 1997; Pugh *et al*., 1997) and the other is thiol-redox regulation of the CAD activity by the thioredoxin and Ref-1 system leading to the interaction with CBP. Prior to transactivation of the target genes in the nuclei, it has recently been known that thioredoxin, HLF and HIF1α have to be activated in response to hypoxia to undergo nuclear translocation from cytoplasm where they are synthesized (this study; Kallio *et al*., 1998). Taken together, these results indicate that the mechanism of signal transduction of a low oxygen concentration by HLF and HIF1α is a multi-step process.

Materials and methods

Recombinant plasmids

pBOS (Mizushima and Nagata, 1990), pBOS-HIF1α full-length, pBOS-HLF full-length, pSV40promoter-EpoHRE-Luc, pBOS-Arnt full-length, pGBT-HIF1α (aa 1–324), pGBT-HLF (aa 1–322) (Ema *et al*., 1997), pBOS-GALDBD (Ema *et al*., 1996a), pBOS-p300 (Kobayashi *et al*., 1997), pRc/CMV-Ref-1, pCDSRα-thioredoxin ($^{32}S/^{35}S$) (Hirota *et al*., 1997) and pCDSRα-thioredoxin (Tagaya *et al*., 1989) were described previously. pG3E-luciferase, pBOS-HIF1α (ΔC), pBOS-HLF (ΔC), pBOS-FLAG-HIF1α full-length, pBOS-FLAG-HLF full-length, pBOS-HLF C848S and pBOS-HIF1α C774S will be described below. pBOS-Arnt (ΔC) was constructed by introducing termination codon into *Eco*81I site of pBKS-hArnt full and subsequently subcloning into the *Xba*I site of pBOS. pBOS-GALDBD-HIF1α or -HLF deletion mutants were constructed as follows; the corresponding cDNA regions were amplified by PCR and subcloned into the *Sma*I site of pSG424 (Sadowski and Ptashne, 1989) and cDNAs for GALDBD and HIF1α or HLF were excised with *Asp*718 and *Bgl*II to be subcloned into the *Xba*I site of pBOS. Serine mutation was created by PCR with the following primers; 5′-CTCTGATTTAGCATGTAGAC-3′, 5′-GGGTACCTCAGTTAACTTCATCCA-3′, 5′-AGTTATGATTCTGAAGTTAA-3′ and 5′-TTAACTTCAGAATCATAACT-3′ for HIF1α; 5′-GTCAGGCGTGGCCAGTCGAC-3′, 5′-GGTACCTCAGGTGGCCTGGTCCA-3′, 5′-CAGATATGACTCTGAGGTGA-3′ and 5′-TCACCTCAGAGTCATATCTG-3′ for HLF. Underlining indicates mutated sequences for Ser. Various pGBT-HIF1α or -HLF derivatives were constructed by subloning the above deletion mutants into the *Sma*I site of pGBT9 (Clontech).

Cell lines and DNA transfection

HeLa and Hep3B cells were maintained in minimal essential medium (MEM) and Dulbecco's modified Eagle's medium (DMEM) supple-

mented with 10% fetal calf serum (FCS), respectively. The expression plasmids were introduced into the cells by the calcium phosphate co-precipitation method together with a *LacZ*-expressing vector pENL as a standard for normalization as described previously (Ema *et al*., 1996a).

Immunoblot analysis

After 48 h of transfection, cells were washed with PBS and collected with silicon rubber. Whole-cell extracts were prepared by mixing with 10 vols of packed cells of buffer A (10 mM HEPES pH 7.9, 0.1 mM EDTA, 0.4 M NaCl, 0.5% NP-40, 0.2 mM sodium orthovanadate, 50 mM sodium fluoride, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonylfluoride) for 20 min and followed by centrifugation for 20 min at 10 000 *g*. Generally, GAL4DBD–HLF and GALDBD–HIF1α fusion protein was poorly expressed and enriched by the following procedure. Supernatants were incubated with protein A–Sepharose beads pretreated with pre-immune serum in 2 vol. of NETN (100 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl pH 7.5, 0.5% NP-40) for 10 min. After centrifugation at 10 000 *g* for 20 min, the supernatants were mixed with 5 μl of anti-GALDBD monoclonal antibody-conjugated agarose beads (Santa Cruz) for 2 h at 4°C and washed twice with 1 ml of NETN. Two volumes of Laemmli buffer were added and boiled for 2 min, and the supernatant was applied to SDS–PAGE. Western blot using anti-GALDBD rabbit serum was performed according to manufacturer's protocol with enhanced chemiluminescence (ECL)-plus reagent (Amersham).

RT–PCR analysis

Total RNAs were prepared from DNA-transfected HeLa cells according to the published method (Chomcynski and Sacchi, 1987), were treated with DNase I and incubated with MMLV reverse transcriptase and oligo(dT) (100 ng) to synthesize cDNAs in 50 μl of reaction solution. After ethanol precipitation, cDNAs were suspended in 170 μl of H_2O and aliquots (2 μl) were applied to the following amplification protocol of 25 cycles, each of which consists of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The resultant DNAs were resolved by agarose gel electrophoresis.

Purification of FLAG-tagged HLF and HIF1α

pBOS-FLAG-HLF full-length or pBOS-FLAG-HIF1α full-length were transfected into HeLa cells together with pUSVT, a plasmid which expresses SV40 large T antigen to amplify the replication of pBOS plasmid. After 48 h of transfection, the medium was sucked off by aspiration and replaced with methionine-deficient MEM (Gibco-BRL) supplemented with dialysed FBS (10%) and [^{35}S]methionine (ICN) (0.1 mCi/ml medium) in the presence or absence of 200 μM $CoCl_2$. After 4 h, whole-cell extracts were prepared and incubated with anti-FLAG antibody-conjugated Sepharose beads (Kodak) for 2 h. The pellets were washed three times with 1 ml of NETN and extracted with 0.1 mg/ml FLAG peptide (Kodak) in PBS for 20 min on ice. The eluates were electrophoresed by SDS–PAGE and the gel was dried and autoradiographed.

Yeast two-hybrid system

Protein–protein interactions were investigated by the two-hybrid system as described previously (Ema *et al*., 1996a). β-galactosidase filter assay was performed according to the published method (Bartel *et al*., 1994).

GST pull-down experiment

GST–CAD–HLF or GST–CAD–HIF1α fusion plasmid was transfected into *Escherichia coli.* BL21 (Lys) strain and the fusion protein was produced and purified to near homogeneity according to the method described previously (Ema *et al*., 1996b) and dialyzed against the following buffer (10 mM HEPES pH 7.9, 50 mM NaCl, 5% glycerol). GST–CAD–HIF1α (2 μg) was treated with 50 mM *N*-ethylmaleimide or DMSO for 20 min at 37°C and adsorbed to glutathione–Sepharose beads (Pharmacia). [^{35}S]methionine-labeled CBP was incubated with 2 μg of GST–CAD–HLF or HIF1α fusion protein-bound beads at 4°C. After 1 h, the beads were pelleted and washed with 1 ml of NETN three times. The beads were mixed with 20 μl of Laemmli buffer and extraced by boiling for 2 min. The eluted proteins were resolved by SDS–PAGE and autoradiographed.

Indirect immunohistochemistry

Indirect immunohistochemistry using an anti-thioredoxin antibody was performed as described previously (Hirota *et al*., 1997) with the following minor modification. Cy-3 conjugated goat anti-mouse IgG (Amersham) was used as a secondary antibody and the specimens were observed with confocal microscopy (MRC-1024, Bio-Rad).

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