CITED2 controls the hypoxic signaling by snatching p300 from the two distinct activation domains of HIF-1α

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HIF-1α plays a central role in cellular adaptation to hypoxia, and is closely related to the pathogeneses of life-threatening disorders. HIF-1α induces the expressions of numerous hypoxia-induced genes through two transactivation domains; N-terminal TAD (NAD) and C-terminal TAD (CAD). Furthermore, p300 is known to boost CAD-dependent transactivation, and CBP/p300-interacting transactivator with an ED-rich tail 2 (CITED2) inhibits HIF-1α-driven gene expression by interfering with the interaction between CAD and p300. However, few researches have focused on the role of CITED2 in the regulation of NAD activity, and thus, we addressed this point. CITED2 was found to attenuate the hypoxic activations of NAD-dependent and CAD-dependent genes, suggesting that CITED2 negatively regulates both CAD and NAD. Immunoprecipitation analyses showed that NAD interacts with the Cystein/Histidine region (CH) 1 and CH3 domains of p300. Moreover, CH1 and CH3 both were required for NAD-dependent transactivation. Furthermore, CITED2 was found to inactivate NAD by interfering with NAD binding to CH1, but not to CH3. These results indicate that CITED2 inactivates HIF-1α by blocking p300 recruitment by both NAD and CAD. We also found that pVHL inhibits NAD activity regardless of NAD degradation by blocking the interaction between p300 and NAD. Summarizing, NAD was activated by binding to p300, and this was blocked by either CITED2 or pVHL. We propose that pVHL controls NAD during normoxia and that CITED2 controls NAD during hypoxia. Our results provide a new strategy for controlling HIF-1α.

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1. Introduction

To adapt to hypoxia, mammalian cells induce numerous proteins required for erythrogenesis, angiogenesis, and anaerobic energy metabolism. HIF-1 plays a central role in cellular adaptation to hypoxia [1], and a growing body of evidence supports that HIF-1α is closely related to the pathogeneses of life-threatening disorders, such as, neoplasia, ischemia, inflammation, and anemia [2,3]. HIF-1α is a heterodimeric transcription factor consisting of HIF-1α and HIF-1β (also called ARNT), both of which belong to the basic helix-loop-helix (bHLH) Per-ARNT-Sim (PAS) family [4]. Of two subunits, HIF-1α is oxygen-dependently regulated at the post-translational level, via its oxygen-dependent degradation domain (ODDD). HIF-1α ODDD is hydroxylated by prolyl hydroxylases (PHDs), then ubiquitinated by pVHL, and finally degraded by proteasomes. Because oxygen is required for the hydroxylation process, HIF-1α is stabilized during hypoxia [5,6].

HIF-1α has two transactivation domains (TADs), namely, N-terminal TAD (NAD) and C-terminal TAD (CAD). Several authors have pointed out that the TADs have different roles in hypoxic responses by transactivating distinct gene subsets [7]. Based on the responsiveness to factor inhibiting HIF (FIH; a CAD-specific inhibitor), Dayan et al. identified the genes governed by CAD and by NAD [8]. For example, CA9, EPO and VEGF were found to be transcribed by CAD, but PGK1, Eno1, and BNIP3 by NAD. To understand HIF-1-driven gene regulation, we need to investigate how CAD and NAD are regulated by oxygen, but only CAD has been extensively investigated to date.

p300/CBP functions as a critical coactivator during HIF-driven transcription. Briefly, p300/CBP remodels chromatin architecture by acetylating histones, which allows transcriptional regulators to approach DNA [9]. CAD recruits p300/CBP by directly binding to the CH1 domain of p300/CBP. This interaction occurs exclusively under hypoxia because the FIH-mediated Asn803 hydroxylation interferes with the CAD–CH1 interaction under normoxia [10]. In contrast to CAD, the molecular mechanism whereby NAD is regulated is poorly understood. Recently, Poellinger and his colleagues showed that NAD and CAD require CBP for transcriptional activation and that NAD recruits CBP by binding to CH3, rather than to CH1 [11]. Given

Abbreviations: HIF-1α, hypoxia-inducible factor 1α; CITED2, CBP/p300-interacting transactivator with an ED-rich tail 2; FIH, factor inhibiting HIF; VHL, von Hippel–Lindau; NAD, N-terminal transactivation domain; CAD, C-terminal transactivation domain; CH1/2, Cystein/Histidine region 1/2.

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reports on the subject, it appears that p300/CBP facilitates HIF-1α transcriptional activity by being recruited by either CAD or NAD.

CBP/p300 interacting transactivator with an ED-rich tail 2 (CITED2; also called Mrg1 or p35srj) is a transcriptional co-regulator that binds to the CH1 of p300/CBP\(^{[12]}\). It functions as a positive or negative regulator of gene expression, depending on its target proteins. When CITED2 associates with AP-2, it acts as a coactivator by enhancing p300/CBP recruitment to AP-2\(^{[13]}\). In contrast, CITED2 acts as a negative regulator of the HIF-1 signaling. Mechanistically, CITED2 blocks the HIF-1α binding to p300/CBP by competing for CH1 binding, and by so doing functionally represses HIF-1 activity\(^{[14]}\). Interestingly, since CITED2 is substantially induced during hypoxia, it is believed to balance HIF activity in a negative feedback manner. Theoretically, to carry out such a function, CITED2 might control both TADs of HIF-1α. However, studies on CITED2 have focused on its inhibition of CAD, and it has not been determined whether CITED2 regulates NAD activity. Given a recent report showing that NAD, like CAD, recruits p300/CBP\(^{[11]}\), we addressed the possibility that CITED2 regulates NAD-dependent gene regulation.

2. Materials and methods

2.1. Reagents and antibodies

Culture media were purchased from Invitrogen (Carlsbad, CA), and fetal calf serum from Sigma-Aldrich (St. Louis, MO). \([\alpha-32P]CTP\) (500 Ci/mmol) was obtained from NEN Life Science (Boston, MA). Anti-HIF-1α antibody was raised in rabbits against human HIF-1α\(^{[15]}\). CITED2, p300, FIH, β-tubulin, Gal4(DBD), and VP16(TAD) primary antibodies and HRP-conjugated secondary antibodies were purchased from SantaCruz Biotechnology (Santa Cruz, CA); anti-HA-tag antibody from Sigma-Aldrich.

2.2. Cell culture

HEK293T (human embryonic kidney), MCF7 (human breast cancer), and Hep3B (human hepatoma) cell-lines were obtained from American Type Culture Collection (Manassas, VA), and VHL(+/+; −/−) RCC4 (renal cell carcinoma) from the European Collection of Cell Cultures (London). Cells were cultured in Dulbecco’s modified...
Eagle’s medium or α-modified Eagle’s medium, supplemented with 10% heat-inactivated fetal calf serum. Gas tensions in the O2/CO2 incubator were 20% O2/5% CO2 for normoxic incubation or 1% O2/5% CO2 for hypoxic incubation.

2.3. Preparation of plasmids, siRNAs and transfection

Hemagglutinin (HA)-tagged HIF-1α, HA-tagged p300, Gal4-CAD (aa. 776–826 of HIF-1α), Gal4-NAD (aa. 498–603 of HIF-1α), Gal4-NAD P564A mutant, Gal4-NAD L574S mutant, VP16-p300 CH1, and VP16-p300 CH3 plasmids were constructed, as previously described [16]. CITED2 (GenBank ID: NM_006079) plasmid and siRNA were constructed as previously described [17]. The sequences of CITED2 and control siRNAs used are 5′-UGACGGACUUCGUGUGCAATT-3′ and 5′-AUGAACGUGAAUUGCUCAATT-3′, respectively. For transient transfection with plasmids or siRNAs, 40% confluent cells in 60-mm dishes were transfected with plasmids or siRNAs using calcium phosphate or Lipofectamine reagent (Invitrogen). The transfected cells were allowed to be stabilized for 40 h before being used in experiments.

2.4. Reporter and mammalian two-hybrid assays

To examine NAD and CAD activities, Hep3B, MCF7 and HEK293T cells were co-transfected with Gal4-promoter luciferase, Gal4-CAD/NAD, or/and CITED2 siRNA using calcium phosphate or Lipofectamine reagent. To examine the interaction between NAD/CAD and p300, HEK293T cells were cotransfected with Gal4-luciferase reporter, Gal4-NAD/CAD, and VP16-Ch1/Ch3 plasmids. The CMV-β-gal plasmid was included in all cotransfection sets. The final DNA or siRNA concentrations were adjusted by adding pcDNA or control siRNA. After 40 h of stabilization, transfected cells were incubated under normoxic or hypoxic conditions for 16 h, and then lysed to determine luciferase. β-gal activities were measured to normalize transfection efficiencies.

2.5. Immunoblotting and immunoprecipitation

Cell lysates were separated on 6.5–12% sodium dodecylsulfate (SDS)/polyacrylamide gels, and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). Membranes were blocked with 5% skim milk for 1 h and then incubated overnight at 4 °C with a primary antibody (1:1000). Membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 h, and stained using the ECL Plus kit (Amersham Biosciences, Piscataway, NJ). To analyze protein interactions, cell lysates were incubated with anti-Gal4 or anti-p300 overnight at 4 °C, and the immune complexes were precipitated with protein A/G beads (Santa Cruz, CA) for 4 h. The precipitated proteins were eluted in the denaturing SDS sample buffer and subjected to SDS-PAGE and immunoblotting.

2.6. Semi-quantitative RT-PCR

mRNA levels were quantified using a highly sensitive semi-quantitative RT-PCR. Total RNAs, extracted using TRIZOL (Invitrogen),
were reverse-transcribed at 46 °C for 20 min, and the cDNAs were amplified over 15–22 PCR cycles (95 °C for 20 s, 50 °C for 30 s, and 70 °C for 1 min) in a 20 μl of reaction mixture containing 5 μCi of [α-32P]dCTP. The PCR products were electrophoresed on a 4% polyacrylamide gel, and dried gels were autoradiographed. PCR primers were designed as shown in Table 1.

2.7. Data analyses

Reporter data were obtained from four independent experiments and analyzed using Microsoft Excel 2007 software. The results were expressed as means and standard deviations, and statistically analyzed by two-sided Mann–Whitney U test. Significant differences between groups were considered when p value was less than 0.05. Immunoblotting, immunoprecipitation, and RT-PCR analyses were performed three times, and representative data were shown in figures.

3. Results

3.1. CITED2 represses either CAD- or NAD-dependent genes during hypoxia

Hep3B cells transfected with CITED2 plasmid or siRNA, were incubated under normoxic or hypoxic conditions for 16 h. Although cell numbers were somehow reduced after hypoxic incubation, the manipulations of CITED2 expression did not affect cell viability (Fig. S1.A). As previously reported [8], we classified HIF-1 target genes into two groups of CAD- and NAD-dependent genes and analyzed their mRNA levels using a highly sensitive semiquantitative RT-PCR technique. The hypoxic expressions of CAD-dependent genes (CA9, EPO and VEGF) were repressed by CITED2 overexpression and enhanced by CITED2 knock-down. Likewise, NAD-dependent genes (PGK1, ENO1 and BNIP3) were also negatively regulated by CITED2 (Fig. 1). We also confirmed that two house-keeping genes, HIF-1α and FIH are constantly expressed and their expressions were neither altered during hypoxia, nor by CITED2 plasmid or siRNA (Fig. S2). These findings support our hypothesis that NAD activity is also controlled by CITED2.

3.2. CITED2 represses the transcriptional activities of CAD and NAD

To confirm the effects of CITED2 on HIF-1α TADs, we analyzed the activities of Gal4-CAD and Gal4-NAD reporters. Hep3B, MCF7 and HEK293T cells were transfected with CITED2 plasmid or siRNA. We found that the activities of both reporters were significantly inhibited by CITED2 overexpression (Fig. 2.A). To examine whether CITED2 controls TAD activities endogenously, we examined the effects of CITED2 siRNA on CAD and NAD activities, and found that both CAD and NAD are noticeably activated by CITED2 knock-down (Fig. 2.B). In these samples, ectopic expression and knock-down of CITED2 were confirmed by checking CITED2 protein levels. Moreover, ectopic expressions of Gal4-CAD and Gal4-NAD were not significantly altered during hypoxia, nor by CITED2 plasmid or siRNA (Fig. S2). To confirm the NAD control by CITED2, we checked if CITED2 plasmid and siRNA regulate NAD activity in a dose-dependent manner, and found their dose-dependent effects on NAD activity (Fig. S3). These results indicate that CITED2 negatively regulates both TADs.

3.3. CITED2 inactivates NAD at a step after the prolyl-hydroxylation of NAD

NAD at the C-terminal part of ODDD includes a Pro564 residue that is targeted by PHDs. Indeed, To and Huang demonstrated that PHD2 binds to NAD and by so doing, represses the transcriptional activity of NAD [16]. To examine whether NAD inactivation by CITED2 is not attributed to the action of PHD, we analyzed the activities of NAD/P564A and NAD/L574S, both of which are not targeted by PHDs. As was expected, both mutants were constitutively active even in normoxia and were slightly activated in hypoxia (Fig. 3.A). Furthermore, both mutants were inactivated by CITED2 overexpression (Fig. 3.B), and more activated by CITED2 knock-down (Fig. 3.C). In the samples, the expression or knock-down of Gal4-NADs or CITED2 was verified by checking the levels of these proteins (Fig. S4). These results indicate that CITED2 repressed NAD activity at a step after PHD targets NAD.

3.4. CITED2 competes with NAD for p300 binding

CAD recruits p300 at its target genes and this boosts CAD-driven transcription [18]. To examine whether NAD also recruits p300, we co-expressed HA-p300 and Gal4-NAD in HEK293T cells. NAD was found to associate with p300 under hypoxic or desferrioxamine-treated condition, but not under normoxia (Fig. 4A). Given that the input levels of Gal4-NAD were not altered substantially, the hypoxic binding of NAD-p300 is unlikely to be due to increased expression of Gal4-NAD. We also found that CITED2 attenuates NAD binding to CAD.

Fig. 4. CITED2 interferes with p300 binding to NAD under hypoxia. p300 binding to NAD was examined using immunoprecipitation and immunoblotting analyses. (A) Gal4-NAD plasmid was co-transfected with HA-p300 into HEK293T cell. On 24 h after transfection, cells were incubated under hypoxic conditions or treated with 130 μM desferrioxamine (DFO) for 8 h. Cell extracts were immunoprecipitated with anti-HA affinity gel for 4 h, and coprecipitated Gal4-NAD was analyzed using anti-Gal4 antibody. (B) HEK293T cells were co-transfected with Gal4-NAD, and HA-p300 and CITED2 (1, 2, and 4 μg). The interaction between Gal4-NAD and p300 was analyzed by immunoprecipitation and immunoblotting. Each protein amount was estimated using the Imagej program (NIH, USA), and the ratio of co-precipitated Gal4-NAD to its input was calculated (% of Input).
p300 in a gene dose-dependent manner (Fig. 4.B). Taken together, these results suggest that NAD and CAD interact with p300 independently and that CITED2 inhibits both interactions.

3.5. NAD binds to both CH1 and CH3 of p300

To examine which one, of CH1 and CH3, binds to NAD, we checked protein interactions using a HIF-1α mutant lacking NAD or CAD (designated HIFΔNAD and HIFΔCAD, respectively). Consequently, CH1 coprecipitated with HIF-1α, HIFΔNAD, and HIFΔCAD (Fig. 5.A). However, CH3 did not associate with HIFΔNAD, while it did with HIF-1α and HIFΔCAD (Fig. 5.B). We rechecked NAD–CHs interactions using the minimal domain of NAD, and found that CH1 and CH3 both associates with NAD (Fig. 5.C). To recheck the interactions, we examined which CH domain competes with p300 for NAD binding. As expected, CH1 and CH3 both inhibited p300 binding to NAD (Fig. 5.D). These results constantly indicate that NAD interacts with p300 by simultaneously binding to CH1 and CH3. In addition, given that NAD–p300 complex was dissociated by either CH1 or CH3, two CH domains are likely to tighten the NAD–p300 interaction cooperatively.

3.6. CITED2 inactivates NAD by interfering with the NAD–CH1 interaction

To check NAD–CHs interactions functionally, we performed mammalian two-hybrid assays. Gal4-NAD was activated in hypoxia, and this activity was significantly stimulated by both VP16-CH1 and VP16-CH3 (Fig. 6.A). This result further supports the notion that NAD interacts with CH1 and CH3. Using this system, we investigated how CITED2 represses NAD activity. The CH1-dependent activity of NAD was noticeably attenuated by CITED2 in both normoxic and hypoxic conditions (Fig. 6.B, left), whereas in contrast, CH3-dependent activity remained high and was further induced in hypoxia even after CITED2 overexpression (Fig. 6.B, right). In the samples, the levels of expressed proteins were verified by immunoblotting (Fig. S5). These findings show that CITED2 represses NAD activity by specifically interfering with NAD–CH1 binding, but not with NAD–CH3 binding. Given that CH1 and CH3 both are required for the interaction between p300 and NAD, CITED2 blockage of the NAD–CH1 interaction may be sufficient to dissociate p300 from NAD.

3.7. pVHL functionally inhibits NAD

CITED2 is induced during hypoxia and functions to set back NAD- and CAD-dependent transcriptions by binding CH1. Therefore, CITED2 might not play a central role in controlling HIF-1α during normoxia. The oxygen-dependent regulation of HIF-1 activity is mainly due to the oxygen-dependent degradation of HIF-1α. In addition, the function of CAD is also limited by the FIH oxygen sensor under aerobic conditions. This double-locking system ensures the oxygen-dependent regulation of CAD, and if so, NAD should be also double-locked under aerobic conditions. Yet, little is known about the oxygen-dependent regulation of NAD. Regarding this point, we considered that the oxygen-dependent targeting of NAD by PHDs and pVHL provides a hint, and thus, we tested the possibility that PHDs and pVHL participate in the regulation of NAD activity. We found that Gal4-NAD activity was enhanced after either hypoxic or desferrioxamine treatment. More interestingly, NAD activity was
significantly inhibited by pVHL overexpression (Fig. 7A) and this was facilitated by VHL knock-down (Fig. 7B). These findings suggest that pVHL negatively regulates the function of NAD. Next, we checked whether the Pro564 hydroxylation is required for the pVHL inhibition of NAD. The activity of NAD_P564A mutant was neither enhanced by hypoxia or desferrioxamine treatment, nor affected by pVHL expression (Fig. 7C, D). We also confirmed that Gal4-NAD and Gal4-NAD P564A expressions were not affected by pVHL expression (Fig. S6). These results suggest that the pVHL action depends on the PHD-mediated hydroxylation of Pro564.

3.8. pVHL interferes with the NAD–CH3 interaction

To understand how pVHL controls NAD activity, we investigated the interactions among NAD, p300, and pVHL. NAD interacted with p300 in VHL knocked-down cells even during normoxia, while it did not in control cells (Fig. 8.A). Also, since Pro564 might be hydroxylated under normoxia regardless of VHL expression, Pro564 hydroxylation per se is unlikely to determine the NAD–p300 interaction. Under hypoxia, NAD interacted with p300, but this interaction was disrupted by pVHL (Fig. 8B). Then, does pVHL compete for NAD binding with CH1 or CH3? When co-transfected with CHs, pVHL did not affect the NAD–CH1 interaction (Fig. 8.C), but pVHL noticeably reduced the NAD–CH3 interaction under hypoxia (Fig. 8.D). To examine whether pVHL interferes with the endogenous interaction between HIF-1α and p300, we compared the HIF-1α–p300 binding in VHL+/+ and VHL−/− RCC4 cells under normoxic conditions. When endogenous HIF-1α was stabilized by MG132 (a proteasome inhibitor), p300 binding to HIF-1α was identified to be substantially enhanced in VHL−/− cells (Fig. 8E). These results suggest that pVHL blocks the NAD–CH3 interaction by preoccupying NAD and by so doing controls NAD-dependent transcription in an oxygen-dependent fashion.

3.9. Proposed mechanism underlying the regulation of HIF-1α activity

On the CAD-dependent genes, p300 interacts with CAD through its CH1 domain and facilitates the CAD-driven transcription. In normoxia, CAD is hydroxylated at Asn803 by FIH and this modification prevents p300 binding to CAD. In hypoxia, CITED2 is induced and sequesters p300 by binding its CH1 domain, leading to a feedback inhibition of CAD activity. On the NAD-dependent genes, p300 interacts with NAD through its CH1 and CH3 domains and transactivates the genes. In normoxia, NAD is hydroxylated at Pro564 by PHDs and recruits pVHL, which interferes with the CH3 binding to NAD. In hypoxia, CITED2 inhibits the CH1 binding to NAD. Accordingly, HIF-1α activity appears to be fine-tuned by the molecular interplay that controls p300 recruitment by NAD and by CAD (Fig. 9).

4. Discussion

Since NAD and CAD express different sets of hypoxia-induced genes, they are believed to play different roles in cellular adaptation to hypoxia. p300/CBP binding to HIF-1α is a critical step in hypoxic gene expression. Therefore, molecules determining this interaction are regarded as potential targets for the control of HIF-related diseases. However, in contrast to CAD, it has not been determined how NAD is regulated, and we addressed this point in the present study. We found that NAD interacts with p300, and that it is activated during hypoxia. In addition, we examined if CITED2 (previously known as an inhibitor of CAD) also inhibits NAD. We found that CITED2 represses NAD function by competing with CAD for p300 binding. Accordingly, the present study provides a better understanding of gene expression determined by HIF-1, and offers a potential strategy to control HIF-1 functionally by inhibiting the p300/CBP interaction.

p300 (like CBP) interacts with numerous transcription factors and functions as a coactivator to facilitate gene expression [19,20]. p300 contains NR, CH1, KIX, Bromo, CH2, HAT, CH3, and Q-rich domains [21], and of these, the CH1–3 domains provide interacting sites for transcription factors and have distinct binding partners. As was mentioned above, CH1 is known to bind to CAD and CITED2 specifically [14]. Furthermore, in the present study, NAD was found to interact with CH1 and with CH3. Since NAD–p300 binding was dissociated by the expression of either CH1 or CH3 peptide, CH1 and CH3 co-binding might be required for the NAD–p300 association. Given that Ets, p53, and Skip2 bind to both CH1 and CH3 [19,22,23], NAD binding to both regions is not surprising. However, our findings contradict a recent report. Ruas et al. carried out immunoprecipitation and fluorescence resonance energy transfer (FRET) analyses, and found that NAD interacts with CH3 of CBP [11]. According to the results obtained, CH1 also interacted with NAD, although this interaction was relatively weak as compared with the CH3 interaction. However, in this previous study, CH domains of CBP and mouse HIF-1α were used, whereas we used CH domains of p300 and human HIF-1α constructs. Accordingly, we believe that these differences between plasmids might have been responsible for the result differences.

In addition to protein stabilization, the activation of HIF-1α is critical for hypoxic signaling. It is well known that CAD activity is regulated oxygen-dependently through the FIH-mediated hydroxylation of Asn803 [24], and in this previous study, it was shown that NAD
activity is also regulated by oxygen level. Since NAD is included in the domain degradable by oxygen, the stabilization of this domain has been regarded to contribute to the hypoxic activation of NAD. This means that no specific mechanism other than protein stabilization has been considered in terms of understanding the hypoxic activation of NAD. In the present study, despite no substantial change in Gal4-NAD level, Gal4-NAD activity was significantly enhanced under hypoxic conditions. Furthermore, the interaction between Gal4-NAD and p300 was enhanced by hypoxia and by deferoxamine. Accordingly, CAD and NAD both are likely to be functionally regulated along oxygen tension.

Given that HIF-1α regulates the transcriptions of key tumor-promoting factors, HIF-1α is generally regarded as one of the most compelling targets for cancer therapy [25,26]. Indeed, many lines of clinical and experimental evidence support the critical roles played by HIF-1α in tumor promotion and metastasis [27]. In addition, it has been reported that the anti-angiogenic effects of many anticancer drugs are attributable to HIF-1α inhibition [28] and that HIF-1α inhibitors have anticancer effects in cultured cells and in vivo [26]. Here, we focused on the inhibitory role of CITED2 in HIF-1α-driven gene expression. These observations and findings beg the question as to whether CITED2 is a possible target for cancer therapy, and a few authors have suggested this possibility. For example, proteasome inhibitors, which are emerging anticancer agents, stabilize CITED2 and by so doing block the hypoxic activation of HIF-1α, which may be associated with the anti-angiogenic effects of these inhibitors [17,29]. In addition, CITED2 level has been reported to be negatively related to colon cancer cell invasiveness and with poor outcomes in breast cancer [30,31]. Thus, CITED2 could be a marker of good prognosis in some cancer patients, and its activation provides a potential strategy for cancer therapy. However, this putative role of CITED2 cannot be established by other than well-designed, large-scale clinical studies.

In terms of the regulation of NAD, a previous report demonstrated that PHD2 participates in its inhibitory regulation and in the degradation of HIF-1α [16]. NAD activity was enhanced during hypoxia regardless of protein stabilization, but this hypoxic activation was not shown by P564A and L573S NAD mutants. Furthermore, the NAD-dependent gene PGK1 was up-regulated in PHD2 knocked-down cells. Although prolyl-hydroxylation was identified to be involved in functional regulation of NAD, the mechanism underlying the oxygen-dependent regulation of NAD was not determined. In particular, the role of p300/CREB-binding protein in NAD regulation was not addressed. Nevertheless, this previously report provides a hint that the PHD-pVHL axis might be involved in NAD regulation, and the present study demonstrates interplay between NAD, pVHL, and p300.

Summarizing, we found that p300 interacts with two activation domains of HIF-1α and subsequently facilitates the gene expressions driven by these two domains. CITED2 snatches p300 from these two domains by binding to CH1 of p300, which leads to the downregulation of gene subsets activated differentially by the two domains. In terms of its binding with p300, NAD differs from CAD; that is, CH1 and CH3 both are required for the p300–NAD interaction, whereas CH1 alone is sufficient for the p300–CAD interaction. We believe that these results provide a deeper understanding of the molecular mechanism underlying NAD-driven gene expression and of the means by which CITED2 controls the transcriptions of HIF-1α target genes.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamcr.2011.08.018.
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