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Modulation of p300 binding by posttranslational modifications of the C-terminal activation domain of hypoxia-inducible factor-1α

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Abstract **Posttranslational modifications of hypoxia-inducible factor-1α (HIF-1α) influence HIF-mediated transcription, likely by affecting binding to p300/cAMP-response element-binding protein (CBP). To systematically analyze the HIF-1α–p300/CBP interaction, we developed a fluorescence polarization-based binding assay, employing fluorescein-labeled peptides derived from the C-terminal transactivation domain (C-TAD) of HIF-1α. After optimized for effectively capturing p300/CBP, the assay was utilized for evaluating direct effects of posttranslational modifications of the HIF-1α C-TAD on p300 binding. The results demonstrated that asparagine hydroxylation and *S*-nitrosylation of HIF-1α decrease p300 binding, while its phosphorylation does not affect p300 binding, which was reconfirmed by competitive inhibition analyses using mutant peptides.**

Keywords: Hypoxia-inducible factor-1α–p300/CBP; HIF-1α–p300/CBP interaction; Fluorescence polarization; Asparagine hydroxylation; *S*-Nitrosylation; Phosphorylation

1. Introduction

Mammalian cells possess the ability to sense and respond to a local decrease in oxygen levels, mediated by hypoxia-inducible factor-1 (HIF-1) [1]. Under hypoxic conditions, inducible HIF-1α is accumulated in the nucleus where it dimerizes with constitutive HIF-1β, which subsequently activates transcription of target genes [2]. Full transcriptional activity of HIF-1 requires the additional interaction of the C-terminal transactivation domain (C-TAD) of HIF-1α with the cysteine/histidine-rich 1 (CH1) domain of the coactivators p300 and cAMP-response element-binding protein (CBP) [3,4].

In normoxia, proline hydroxylation of HIF-1α signals binding of the von Hippel-Lindau protein (VHL), leading to HIF-1α destruction [5,6]. The HIF-1α–VHL complex further recruits factor inhibiting HIF-1α (FIH-1), which hydroxylates Asn-803 in the C-TAD of HIF-1α, thereby disrupting its interaction with p300/CBP and blocking the HIF-mediated transactivation [7]. In addition to asparagine hydroxylation, other posttranslational modifications of residues in C-TAD have been proposed to influence the fine-tuning of HIF-1α function [8]. *S*-Nitrosylation of Cys-800 in HIF-1α has been shown to increase HIF-1 transcriptional activity possibly by enhancing its p300 binding [9], while some conflicting effects of nitric oxide on hypoxia-induced transactivation have been reported [10]. Phosphorylation of HIF-1α can also modulate its transcriptional activity. The hypoxia-induced activation of mitogen-activated protein kinase has been suggested to phosphorylate HIF-1α, which appears to increase its p300 binding but is not correlated with its transcriptional activity [11]. However, the phosphorylation sites responsible for HIF-1α nuclear accumulation have been identified to reside far from the C-TAD [12]. On the other hand, Thr-796 in C-TAD has been indicated as a candidate for phosphorylation possibly by casein kinase 2 (CK2) [13]. Despite the abundance of transcription activity data affected by posttranslational modifications of the HIF-1α C-TAD, scattered information is available for the direct effects of such modifications on its p300/CBP binding.

We have previously developed a HIF–VHL interaction assay, which could be utilized for monitoring prolyl hydroxylation [14]. Here, we have employed fluorescein-labeled peptides derived from the C-TAD to set up a similar fluorescence polarization (FP)-based binding assay, which has been utilized for systematically evaluating the HIF–p300/CBP interaction, and examining the effects of post-translational modifications of the C-TAD peptide on p300 binding.

2. Materials and methods

2.1. Materials

S-Nitroso-*N*-acetylpenicillamine (SNAP), Sephadex-G15, and thrombin were purchased from Sigma–Aldrich (St. Louis, USA), CK2 from New England Biolabs (Beverly, USA), and streptavidin from Calbiochem (Darmstadt, Germany). To prepare a biotinylating reagent for quantification of *S*-nitrosylation, biotin-Cys-NH_2 was synthesized on Rink Amide™ resin (NovaBiochem) using Fmoc-Cys (25 μmol), followed by addition of 12 mg 1,11-bis-maleimidotriethyleneglycol (Pierce) to the 1.5 ml solution of biotin-Cys-NH_2 (4 mg) in PBS/DMSO (1:2 vol/vol). After the mixture was stirred at room temperature overnight, the biotinylating reagent was purified by reverse-phase HPLC, and characterized by MALDI-TOF mass spectrometry.

2.2. Peptide preparation

Fluorescein-labeled peptides containing the C-TAD of human HIF-1α were synthesized by conjugating fluorescein with the N-terminal insertion of an aminocaproic acid linker (AnyGen, KwangJu, Korea).

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Abbreviations: CBP, cAMP-response element-binding protein; CK2, casein kinase 2; C-TAD, C-terminal transactivation domain; FIH-1, factor inhibiting HIF-1α; FP, fluorescence polarization; HIF, hypoxia-inducible factor; VHL, von Hippel-Lindau protein

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The synthesized peptides containing residues 776–826, 786–826-, 788–822, and 776–813 were denoted as F-HIF-1α-(776–826), F-HIF-1α-(786–826), F-HIF-1α-(788–822), and F-HIF-1α-(776–813), respectively.

For mutant peptides, human HIF-1α-(786–826) and its single mutation products prepared by PCR were subcloned into pGEX-2T-1 (Amersham Biosciences) and overexpressed in *E. coli* BL21(DE3). After purification of the GST fusion proteins using glutathione–Sepharose (Amersham Biosciences) and GST removal by thrombin treatment, the resulting peptides were purified using reverse-phase HPLC and confirmed by MALDI-TOF mass spectrometry.

2.3. Protein expression and purification

The truncated human CBP (amino acids 1–450) subcloned in pGEX-KG, and the truncated human p300 (amino acids 300–520) and the full-length human FIH-1 (amino acids 1–349) subcloned in pGEX-4T-1 (Amersham Biosciences) and pET-28a (Novagen), respectively, were overexpressed in *E. coli* BL21(DE3). GST-CBP and GST-p300 were purified on glutathione-Sepharose, and His-FIH-1 on Ni-NTA agarose (Qiagen). The purified fusion proteins were confirmed by SDS–PAGE and quantified by Bradford assay (Bio-Rad).

2.4. Asparagine hydroxylation by FIH-1

Five micromolar F-HIF-1α-(786–826) was incubated with recombinant His-FIH-1 (0.7 μg/μL) in hydroxylation reaction buffer (20 mM Tris–HCl, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$) containing 400 μM ascorbic acid and 100 μM α-ketoglutarate. After incubation for 2 h at room temperature, the reaction mixture was passed through $ZipTip_{C18}$ (Millipore, USA) for desalting, followed by elution by adding α-cyano-4-hydroxycinnamic acid in acetonitrile/water containing 0.1% TFA (1:1 vol/vol). The eluted peptide solution was then transferred to a MALDI sample plate and MALDI-TOF measurements were performed with a Voyager analyzer (Applied Biosystems). For binding assays, the hydroxylated peptide was purified by reverse-phase HPLC.

2.5. S-Nitrosylation by SNAP

Thirty micromolar F-HIF-1α-(786–826) and F-HIF-1α-(786–826)[C800A] containing Ala substitution for Cys were incubated with 2 mM SNAP in 50 mM Tris, pH 8.0 for 30 min at 30 °C. After excess SNAP was removed using a Sephadex G-15 resin, *S*-nitrosylation of the peptide was determined by absorbance measurements of the nitrosothiol moiety [15], or by FP measurements upon addition of streptavidin to the peptides reacted with the biotinylating reagent (300 μM) for 2 h at room temperature. The *S*-nitrosylated peptide was obtained by separating from the biotinylated one on reverse-phase HPLC for binding assays.

2.6. Phosphorylation with kinases

Phosphorylation reactions were performed with CK2 (500 U) in 50 μL of kinase reaction buffer (25 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 2 mM DTT, 0.1 mM Na_3VO_4) containing 100 μM ATP with or without [γ-^{32}P] ATP using 3 μM F-HIF1α-(786–826) or F-HIF1α-(788–822) as substrates for 30 min at room temperature for radioactive assays, or for 3 h at 30 °C for MALDI-TOF analysis. For binding assays, the phosphorylated product was purified by reverse-phase HPLC after the reaction at room temperature overnight.

2.7. Binding assays

Fluorescein-labeled peptides (100 nM) were mixed with GST-CBP or GST-p300 in EBC buffer (50 mM Tris, pH 8.0, 120 mM NaCl, and 0.25% Nonidet P40) at room temperature, followed by FP measurements using an LS50B fluorimeter (Perkin-Elmer). Representative binding data of at least duplicate assays are shown in figures where error bars are omitted since they are within the symbol points, and the data for at least three independent experiments were analyzed using KaleidaGraph software as described previously [14].

3. Results and discussion

3.1. FP-based measurements of the interaction of HIF-1α peptides with p300/CBP

The CH1 domain of p300/CBP that interacts with HIF-1α is stabilized by Zn^{2+}-binding while the metal-free CH1 is disordered [16]. Therefore, the importance of Zn^{2+} in p300/CBP preparations was first tested for their binding to the HIF-1α-derived peptides by preparing GST-p300 and GST-CBP proteins in the absence or presence of $ZnSO_4$. For the binding assays, fluorescein-labeled F-HIF-1α-(786–826) and F-HIF-1α-(776–826) were utilized for GST-p300 and GST-CBP, respectively, since the C-terminal 41 residues 786–826 appear to be important for p300 binding [7], and the 51 residues 776–786 for CBP binding [17]. Due to the size difference, FP of F-HIF-1α-(776–826) (Fig. 1A) was higher than that of F-HIF-1α-(786–826) (Fig. 1B). Addition of GST-CBP (Fig. 1A) and GST-p300 (Fig. 1B) to the peptides increased FP significantly to the similar values irrespective of the $ZnSO_4$ inclusion in protein preparations, indicating that Zn^{2+} in the culture media is present enough to complex with the recombinant proteins. Since excess Zn^{2+} might induce misfolding of p300/CBP [18], $ZnSO_4$ was excluded in p300/CBP preparations. It should be noted that GST incubated with the fluorescein-labeled peptides did not show any change in FP (data not shown), confirming no non-specific binding.

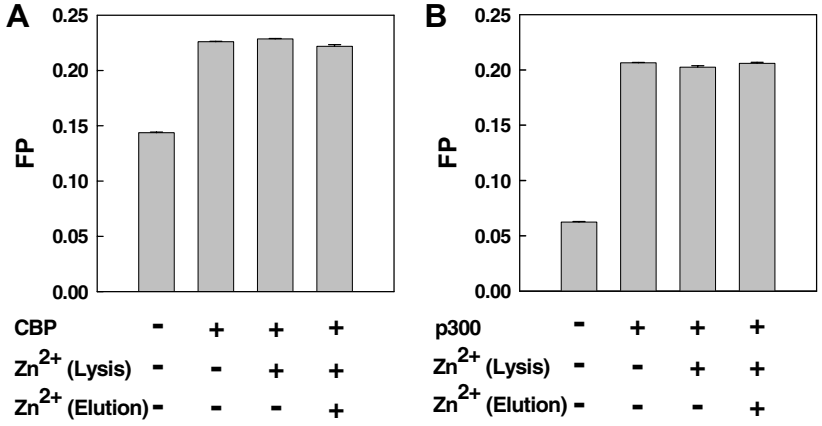

Fig. 1. Effects of Zn^{2+} in the preparation of p300/CBP proteins on their interactions with fluorescein-labeled HIF-1α peptides. In recombinant GST-CBP and GST-p300 preparations, 0.1 mM $ZnSO_4$ was omitted or included either in lysis buffer alone or in lysis and elution buffers. FP changes were measured for (A) F-HIF-1α-(776–826) at 100 nM incubated with or without 800 nM GST-CBP, and (B) F-HIF-1α-(786–826) at 100 nM incubated with or without 800 nM GST-p300.