Molecular Mechanism of Hypoxia-inducible Factor 1α-p300 Interaction

A LEUCINE-RICH INTERFACE REGULATED BY A SINGLE CYSTEINE*

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Hypoxia-inducible factor 1α (HIF1α) plays a pivotal role in embryogenesis, angiogenesis, and tumorigenesis. HIF1α-mediated transcription requires the coactivator p300, at least in part, through interaction with the cysteine- and histidine-rich 1 domain of p300. To understand the molecular basis of this interaction, we have developed a random mutagenesis screen in yeast approach for efficient identification of residues that are functionally critical for protein interactions. As a result, four residues (Leu-795, Cys-800, Leu-818, and Leu-822) in the C-terminal activation domain of HIF1α have been identified as crucial for HIF1 transactivation in mammalian systems. Moreover, data from residue substitution experiments indicate the stringent necessity of leucine and hydrophobic cysteine for C-terminal activation domain function. Likewise, Leu-344, Leu-345, Cys-388, and Cys-393 in the cysteine- and histidine-rich 1 domain of p300 have also been shown to be essential for the functional interaction. We propose that hypoxia-induced HIF1α-p300 interaction relies upon a leucine-rich hydrophobic interface that is regulated by the hydrophilic and hydrophobic sulfhydryls of HIF1α Cys-800.

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The abbreviations used are: HIF1, hypoxia-inducible factor 1; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; CH1, cysteine- and histidine-rich 1; CAD, C-terminal activation domain; RAMSY, random mutagenesis screen in yeast; PCR, polymerase chain reaction; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; GST, glutathione S-transferase.

EXPERIMENTAL PROCEDURES

RAMSY—The interaction between LexA-CAD and B42-CH1 was first determined in the matchmaker LexA two-hybrid system (CLONTECH) after cotransformation into yeast strain EGY48[p80p-lacZ] according to the manufacturer’s instructions. To create overhangs for in vivo homologous recombination, PCR primers for random mutagenesis were designed such that the upstream primer (5′-GGGAGTTTTAAC-CAATTTGTGTTAGA-3′) started 92 base pairs upstream of the EcoRI site of pLexA, whereas the downstream primer (5′-CAGGAAAGAAGT-TACTCAAGAAAGATT-3′) was 157 base pairs downstream of the XhoI site of the vector. The mutagenic PCR reaction was performed in a total volume of 25 μl with 1 μM dCTP, dGTP, and dTTP and 0.2 μM

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Molecular Basis of HIF1α-p300 Interaction

In search of the molecular determinants of HIF1α-p300 interaction, we developed a RAMSY technique that couples PCR-mediated random mutagenesis (37, 38) with a LexA yeast two-hybrid system (Fig. 1). We began the two-hybrid assay by constructing two fusion plasmids; HIF1α-CAD (24), and pLexA-CAD (amino acids 776–826) (16, 17) and pB42-C/H1 (amino acids 311–355) (24) was fused to the Gal4 DNA-binding domain and tested for transcriptional activation in vivo. The CAD region was PCR-amplified for DNA sequencing (37, 38). The mutant products were cotransformed with a gapped pLexA vector into the yeast two-hybrid strain, resulting in in vivo homologous recombination. After appropriate nutritional selection, white colonies indicating loss of CAD-C/H1 interaction were selected and confirmed with their inability to activate the LEU2 gene. DNA sequences were amplified from these clones, and the CAD region was PCR-amplified for DNA sequencing (37, 38). This is presumably due to a lack of necessary factor(s) in yeast. Accordingly, cotransformation with pB42-C/H1 resulted in marked activation of both reporter genes in a galactose-dependent manner (data not shown).

Next, the CAD region was PCR-amplified under a mutagenic condition in which the dATP concentration was reduced to 10% of the total transformants were gathered and tested further for inability to transactivate the LEU2 gene. Only those that were unable to activate both reporters were selected for PCR amplification and DNA sequencing of the mutagenized region.

Compilation of sequencing data from a total of 32 clones revealed that codons Leu-785, Cys-800, Leu-818, and Leu-822 of HIF1α were among the most frequently mutated (Table I, top), indicating the importance of these residues for C/H1 interaction. Of note, in addition to the above clones, 16 more clones contained either premature stop codons or reading frameshifts, and 3 more clones showed no mutations within the CAD presumably because nonsense mutations occurred in the upstream homologous recombination region. Silent mutations were also observed in some clones (data not shown). It is noteworthy that all of these four residues are conserved across various species of cloned HIF1α as well as HIF2α including human, mouse, rat, dog, chicken, Xenopus, and quail (data not shown). Hence we decided to focus on these four residues for in-depth analysis of their role in transcriptional activation in mammalian systems.

Essential Role of the Identified Residues for CAD Transactivation—Initially, CAD and its mutants were fused to a Gal4 DNA-binding domain and tested for transcriptional activation in yeast, in agreement with a previous report (25). This is presumably due to a lack of necessary factor(s) in yeast. Accordingly, cotransformation with pB42-C/H1 resulted in marked activation of both reporter genes in a galactose-dependent manner (data not shown).

RESULTS

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of a Gal4-luc reporter in Hep3B cells. As shown previously (16, 17), CAD exhibited strong hypoxia-induced transcriptional activity (Fig. 2a). By contrast, none of these mutants (L795P, C800R, L818S, and L822S) did so, even though their protein expression levels were similar to that of the wild type (Fig. 2a, inset). Because these mutants were originally identified to be critical for interaction with C/H1 in yeast, the above result implied that CAD transactivation relies on interaction with endogenous p300/CBP. To test this hypothesis, we cotransfected a vector that either expresses C/H1 fused to a VP16 activation domain (VP16-C/H1) or C/H1 fused to a yellow fluorescent protein (EYFP-C/H1) as a tag in the above setting. As shown in Fig. 2b, the C/H1 domain, when fused to EYFP, apparently competed against endogenous p300/CBP for CAD binding, thereby inhibiting transcription. In contrast, VP16-C/H1 further enhanced transcriptional activity of the wild-type CAD but not that of the mutants (Fig. 2c). These results confirm that the residues identified by RAMSY are functionally critical for CAD transcriptional activity in mammalian cells. To provide evidence that this observation is a result of loss of C/H1 binding, we performed GST pull-down experiments in which GST-C/H1 or GST-ΔC/H1 (24) was incubated with in vitro translated Gal4-CAD or the mutants. As expected, wild-type Gal4-CAD interacted with GST-C/H1 but not with GST-ΔC/H1, and furthermore none of the mutants bound to C/H1 (Fig. 2d). Therefore, we conclude that Leu-795, Cys-800, Leu-818, and Leu-822 of HIF1α are functionally indispensable for CAD transcriptional activity in mammalian cells.

**Stringent Necessity of Leucines and Hydrophobic Cysteine for CAD Activity**—It appears that CAD requires hydrophobic leucines for C/H1 binding; replacement with the hydrophilic residues abolished its transcriptional activity. To test the stringency of these leucines, we asked whether different hydrophobic residues such as valine and alanine could mimic leucine. Interestingly, individual replacement of Leu-795 and Leu-822 with valine abrogated CAD transcription (Fig. 3, a and c), whereas L818V substitution showed only modest reduction. However, alanine substitutions of these leucines abolished CAD function. These findings argue against the loss of CAD activity as a result of global structural disruption but rather suggest that these leucines engage in direct contact with the C/H1 domain. In keeping with this notion, the impairment of CAD function was in good agreement with loss of C/H1 binding in vitro, with the exception of L795V (Fig. 3, b and d). Taken together, these results suggest the stringent requirement of these leucines for CAD transcriptional activity.

Cys-800 has been suggested to be a target of redox modulation of HIF1α transactivation because serine substitution inhibited CAD activity and p300/CBP binding (25), but this notion is apparently not supported by alanine replacement, showing a modest effect (36). To gain a definitive understanding of the functional role of Cys-800, we mutated Cys-800 to structurally close but biochemically distinct residues including serine, threonine, aspartate, asparagine, alanine, and valine. Remarkably, substitutions with the first four hydrophilic residues exhibited invariable loss of CAD transcriptional activity (Fig. 3, e and f) and C/H1 binding (data not shown). However, hydrophobic residue substitutions not only retained CAD function, but valine replacement further increased CAD activity (Fig. 3f), whereas C/H1 binding activity and protein expression levels were equivalent to those of the wild type (data not shown). Thus, we conclude that HIF1α Cys-800 plays a regulatory role for C/H1 binding and HIF1 transactivation by the reversible change between -SH (hydrophobic) and -S· (hydrophilic) groups.

**Molecular Determinants of HIF1α-p300 Interaction**—To verify the role of these identified residues in HIF1α-mediated transcription, we introduced mutations to a full-length HIF1α and tested their effects on a HIF1α-responsive luciferase reporter. Consistent with the results obtained from Gal4 fusions, all of the individual mutations resulted in a significant decrease in HIF1 transcription in COS7 cells (Fig. 4f). Furthermore, unlike wild-type HIF1α, these mutants failed to bind to C/H1 in a GST pull-down assay (data not shown). It is noteworthy that in addition to CAD, HIF1α possesses another activation domain upstream of CAD, namely N-terminal activation domain (16, 17) (Fig. 4e), but its transcriptional activity appears to be less potent in the absence of CAD (39). Moreover, we previously showed that CAD is sufficient for HIF1-mediated transcription in the absence of N-terminal activation domain (18, 40). Consistently, introduction of L795V and L822V mutations to an N-terminal activation domain-deleted HIF1α significantly reduced HIF1 transcriptional activity (Fig. 4c). These results

### Table I

**Critical codons identified by RAMSY**

| Codon | Substitutions | Single hit ratio |
|-------|---------------|-----------------|
| HIF1α |               |                 |
| L795  | P (5), Q (3), R (1) | 5/9             |
| C800  | R (9)          | 7/9             |
| L818  | S (4)          | 3/4             |
| L822  | S (5)          | 3/5             |
| p300  |               |                 |
| L344  | P (5)          | 2/5             |
| L345  | P (7)          | 2/7             |
| C388  | R (3), W (1)   | 1/4             |
| C393  | R (4)          | 2/4             |

Numbers in parentheses represent frequencies of particular residue substitutions. Single hit ratio equals single mutation frequency divided by total number of mutations.
lend further support to the notion that Leu-795, Cys-800, Leu-818, and Leu-822 of HIF1α are essential for HIF1α transactivation.

The effectiveness of RAMSY warranted investigation of the C/H1 domain. Following random mutagenesis of the C/H1 domain in B42-C/H1, compiled sequencing data from a total of 57 clones revealed that codons Leu-344, Leu-345, Cys-388, and Cys-393 of p300 were among the most frequently mutated (Table I, bottom). Consistently, an independent study based on sequence alignment also demonstrated recently that these two cysteines are required for HIF1α interaction and for the integrity of a zinc bundle structure within the α-helical C/H1 domain (33). The biological function of the four residues was subsequently evaluated by testing whether mutations of these residues would interfere with endogenous p300/CBP binding to CAD in Hep3B cells, as shown in Fig. 2B. In contrast to the wild-type EYFP-C/H1, these mutant fusions failed to inhibit Gal4-CAD activity (Fig. 4d), implying that the identified p300/CBP residues are required for HIF1α binding and transactivation. Consistently, because the C/H1 domain utilizes distinct residues to interact with a variety of factors (33, 34), overexpression of the C/H1 mutants might compete for binding to the competitive inhibitors of HIF1α transactivation, e.g. p35srj, thereby releasing more endogenous p300/CBP and in turn stimulating Gal4-CAD transcriptional activity.

**DISCUSSION**

We have successfully employed the RAMSY technique to identify the molecular determinants of the HIF1α-p300 interaction, which provides a molecular basis for understanding the mechanisms underlying HIF1α activation. As a result, we propose that hypoxia-induced HIF1α-p300 interaction requires a leucine-rich hydrophobic interface that is regulated by the reversible change between hydrophobic and hydrophilic Cys-800 of HIF1α. This hypothesis is in part based upon the results from Cys-800 replacement with structurally similar but biochemically distinct residues including valine, and is consistent with the functional role of the reducing factor Ref-1 in CAD transcriptional activation (25, 35). Our results have also ruled out the possibility that CAD transcriptional activity requires Cys-800 to form a disulfide bond with the C/H1 domain of p300/CBP.

It is noteworthy that valine substitution markedly increased normoxic CAD activity, thereby decreasing hypoxic inducibility of CAD. However, the hypoxic induction still remained (Fig. 3f), indicating the possibility of additional mechanisms contributing to hypoxia-induced CAD-C/H1 interaction or CAD transcriptional activity. Interestingly, among all the clones of the CAD mutants sequenced, none of the mutations occurred at codons 751–783 of the conserved RLL sequence that was reported to be critical for hypoxic induction in HIF2α (36). This difference might be resolved by sequencing a larger population of CAD mutants even though the result could be unpredictable. In theory, the RLL sequence had the same mutation probability as the identified residues because of random mutagenesis (37). Alternatively, it is possible that functionally defective mutants in mammalian systems are functional in yeast and therefore cannot be detected by RAMSY. In addition, p300/CBP binds the nuclear hormone receptor coactivator SRC-1, which has been shown recently to be an active part of the HIF1α transcriptional complex (35). The involvement of SRC-1 might...
explain why the L795V mutation did not affect C/H1 binding in vitro but significantly inhibited CAD transactivation (Fig. 3z), because such mutation might pose a steric hindrance to SRC-1 binding in vivo, thereby interfering with CAD function. Likewise, it is conceivable that the “superactive” nature of the C800V mutation is a result of favored SRC-1 binding in addition to the unaffected p300/CBP binding.

We have shown that RAMSY, similar to the reverse two-hybrid system (41), is a simple, efficient, and reliable approach by which molecular determinants of two interacting mammalian proteins can be uncovered readily in yeast. We demonstrated its efficiency to pinpoint the most critical (if not all) residues involving protein-protein interactions; single (instead of multiple) mutations of the identified residues abrogate the protein function, indicating a critical role for these residues. As the yeast two-hybrid system has been widely employed in the last decade, RAMSY should provide a broadly applicable means for the efficient revelation of the molecular basis of a wide range of protein-protein interactions and in turn lead to a better understanding of the mechanisms underlying various biological processes.

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