Interaction of the TAZ1 Domain of the CREB-Binding Protein with the Activation Domain of CITED2

REGULATION BY COMPETITION BETWEEN INTRINSICALLY UNSTRUCTURED LIGANDS FOR NON-IDENTICAL BINDING SITES*

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The TAZ1 domain of the homologous transcriptional coactivators CREB-binding protein (CBP) and p300 forms a complex with CITED2 (CBP/p300-interacting transactivator with ED-rich tail), inhibiting the activity of the hypoxia inducible factor (HIF-1α) and thereby attenuating the cellular response to low tissue oxygen concentration. We report the NMR structure of the CBP TAZ1 domain bound to the activation domain of CITED2. The structure of TAZ1, consisting of four α-helices (αα-αα) stabilized by three zinc atoms, is very similar in the CITED2 and HIF-1α complexes. The activation domain of CITED2 is unstructured when free and folds upon binding, forming a helix (termed αL) and an extended structure that wraps around TAZ1. The CITED2 αL helix packs in the TAZ1 αα/αα interface, a site that forms weak interactions with the poorly defined amino-terminal α-helix of HIF-1α. CITED2 and HIF-1α both contain a four residue motif, LP(E/Q)L, which binds in the TAZ1 αα/αα/αα/αα junction in each complex. The carboxyl-terminal region of CITED2 forms an extended structure with hydrophobic contacts in the TAZ1 αα/αα interface in the site occupied by the HIF-1α αα helix. CITED2 does not bind at all to the TAZ1 site occupied by the HIF-1α carboxyl-terminal helix. The HIF-1α and CITED2 domains utilize partly overlapping surfaces of TAZ1 to achieve high affinity binding and to compete effectively with each other for interaction with CBP/p300; CITED2 and HIF-1α use these binding sites differently to maintain similar binding affinities in order to replace each other in a feedback loop during the hypoxic response.

The cellular response to hypoxia in mammals involves a complex interplay of a number of transcriptional molecules. The primary response to hypoxia involves the heterodimeric transcription factor called hypoxia-inducible factor 1 (HIF-1), a major factor in the pathology of cancer, heart disease, and stroke (1–3). Under conditions of oxygen deprivation, the HIF-1α subunit recruits the general transcriptional coactivators CBP or p300 to direct the expression of genes necessary for survival (4). Deletion of HIF-1α in mice results in neural and cardiovascular developmental arrest and embryonic death (5). Consistent with the central role of the HIF-1 system in the maintenance of cellular oxygen homeostasis, a variety of mechanisms, including oxidative degradation and transcriptional inactivation, closely regulate the activity of HIF-1α. Under normoxic conditions, low HIF-1α protein levels are maintained by a family of specific prolyl hydroxylases (PHD1–3) that target HIF-1α for ubiquitination and proteasome-mediated degradation. These hydroxylases modify two prolines within conserved LXXLAP motifs in HIF-1α to enhance interaction with the von Hippel-Lindau E3 ubiquitin ligase complex (6–9).

The transcriptional activity of HIF-1α is directly dependent on the interaction of its carboxyl-terminal activation domain (CAD) with the TAZ1 domain, also referred to as cysteine/histidine-rich domain 1 (C/H1), of CBP/p300 (4, 10). FIH-1 (factor inhibiting HIF-1) (11), a second class of oxygen dependent HIF-1α specific regulator, hydroxylates an asparagine residue within the HIF-1α CAD to attenuate the interaction with the coactivator CBP/p300 (12). Recent NMR structures from our laboratory and others highlight the exquisite specificity involved in the formation of the HIF-1α CAD-TAZ1 complex and the nature of the molecular interactions that define the hypoxic switch (13, 14). In the complex, the CBP TAZ1 domain is composed of four α-helices stabilized by three zinc atoms in a fashion similar to that of the isolated CBP TAZ2 domain (15). Upon binding to CBP, the HIF-1α CAD undergoes a folding transition to form three helices, namely a poorly defined amino-terminal helix (αL) that interacts weakly with TAZ1 and two tight-binding helices, αα and αα, all of which wrap around the TAZ1 domain in highly intimate fashion. The TAZ1 domain thus acts as a scaffold, providing extensive hydrophobic surface grooves to accommodate the transcription factor activation domain.

In hypoxia, a third HIF-1α regulatory mechanism is proposed to be mediated by the protein CITED2 (CBP/p300 Inter-
acting Transactivator with glutamate (E) and aspartate (D) rich tail, also termed MRG-1 and p35-raj (16). CITED2 is a ubiquitously expressed nuclear protein that competes with HIF-1α for binding to the CBP/p300 TAZ1 domain. In mice, deletion of CITED2 results in embryonic lethality due to severe cardiac and neural tube defects (17). CITED2 knockouts also exhibit increased expression of vascular endothelial growth factor (VEGF) and other hypoxia-responsive gene products. The severe phenotype of CITED2−/− mice is consistent with the additional function of CITED2 as coactivator for AP-2 transcription factors, which are necessary for neural development (18, 19). Expression of CITED2 is directly induced by HIF-1α, implicating CITED2 as a negative feedback regulator of HIF-1α and a fundamental component of the cellular mechanism for attenuation of the hypoxic response.

Previous domain mapping and mutagenesis experiments have defined the region of interaction with CBP/p300 TAZ1 to be the carboxyl-terminal activation domain of CITED2 (16). As is typical of transcription factor activation domains, the CITED2 CAD contains a relatively large number of residues with acidic and bulky hydrophobic side chains but has no obvious sequence homology with the HIF-1α CAD (Fig. 1). To determine the structural basis of HIF-1α inhibition by CITED2, we have determined the solution structure of the CBP TAZ1 domain bound to the activation domain of CITED2. The structure of TAZ1-CITED2 reveals the commonalities, as well as major differences, between the binding of CITED2 and HIF-1α to TAZ1. As this work was nearing completion, the solution structure of a homologous TAZ1 domain from the p300 coactivator, also bound to human CITED2, was reported (20). Aside from the structural differences imposed by the sequence differences between the TAZ1 domains of CBP and p300, there are significant differences in the lengths of the domain constructs used in the two studies. The structures of the two complexes, TAZ1-HIF-1α and TAZ1-CITED2, together with NMR relaxation data that probe the backbone flexibility, suggest a mechanism of how CITED2 and HIF-1α displace one another from TAZ1.

**EXPERIMENTAL PROCEDURES**

**(Protein Expression and Purification)—**A coexpression plasmid (21) containing mouse CBP TAZ1 (residues 340–439) and human CITED2 (204–269, 220–269) was transformed into *Escherichia coli* BL21(DE3) (darm) and grown in 6-liter minimal medium containing 0.1 mM ZnCl2 and, for isotopic labeling, 15NH4Cl (1 g/liter) and/or 13C-glucose (2 g/liter). Cells were grown at 37°C, induced with 1 mM isopropyl-β-D-thiogalactopyranoside at an OD600 of ~0.8, and left at 15°C for overnight expression. Cells were harvested and resuspended in 150 ml of buffer (10 mM Tris, pH 8.5, 50 mM NaCl, 20 mM dithiothreitol, and 6 M urea) and sonicated, and the cell lysate (160 ml) was diluted to 4 M urea and loaded unto a 10-ml SP-Sepharose attached to a 10 ml Q-Sepharose column (Amersham Biosciences). TAZ1 was eluted from the SP-Sepharose column with a linear gradient of buffer (10 mM Tris, pH 8, 50 mM NaCl, 1 M NaCl, 4 M urea) and further purified through a C4, and a C reverse phase HPLC columns. TAZ1 and HIF-1α CAD were prepared as described previously (13). Final NMR buffer was 10 mM Tris-d1, 10% 2H2O, and 2 mM Na3PO4, pH 6.8.

**NMR Sample Preparation—**The lyophilized CITED2 was dissolved in 10 mM deuterated Tris-d1, adjusted to pH 7, and dialyzed in the same NMR buffer as TAZ1. To prepare the complex, CITED2 and TAZ1 were combined in 20–30 µl increments to give a final volume of 600 µl and a final molar ratio of 1:1.3 of labeled to unlabeled partner. Upon mixing TAZ1 and CITED2, precipitates formed in the NMR tube. The effective complex concentration was then estimated by comparing the NMR signal to a known sample of free protein. NMR samples contained ~400–500 µM complex. NMR samples in 100% H2O were prepared by lyophilizing the deuterated TAZ1 and CITED2 and re-dissolving the proteins in H2O before mixing to form the complexes.

**Circular Dichroism Spectroscopy—**CD spectra were collected at 25°C using an Aviv model 202 CD spectrometer and a 0.2 cm cell. Samples contained 7 µM protein in 3 mM Tris-HCl buffer (pH 7.5).

**Isothermal Titration Calorimetry—**ITC data were collected at 25°C using a Microcal MCS Titration calorimeter. Purified protein samples were dialyzed into ITC buffer (20 mM Tris, pH 6.8, containing 50 mM NaCl and 2 mM dithiothreitol) and centrifuged to remove aggregates. Protein concentrations were measured by a standard bicinchoninic acid protein assay. Titration data were fitted to a one-site model using the Microcal data analysis software ORIGIN 2.3 (Microcal Software, Northampton, MA) and were not adjusted for buffer effects. ITC data were acquired at least three times using different batches of samples on different days to obtain an error estimate for the Kd values reported in Table 1.

**NMR Spectroscopy—**NMR spectra were acquired at 25°C on Bruker DRX600, DRX800, and AVANCE 900 MHz spectrometers, processed using NMRPipe (22), and analyzed using NMRView (23). Resonance assignments for TAZ1 and CITED2 were obtained from heteronuclear
Structure of the CBP TAZ1-CITED2 Complex

RESULTS

The free CITED2 activation domain, residues 220–269, is unstructured in solution as indicated by the absence of secondary structural elements in the circular dichroism spectra (Fig. 2A) and the narrow range of amide proton chemical shifts in the two-dimensional $^1$H-$^1$N HSQC spectrum of $^{15}$N-labeled CITED2 (Fig. 2B). The CD spectrum of the complex (Fig. 2A) shows the formation of additional structure compared with the individual proteins, as indicated by a comparison of the spectrum of the complex with the sum of the spectra of the two components. Upon binding to TAZ1, there is increased dispersion of the CITED2 amide proton chemical shifts, indicating structure formation (Fig. 2B). A longer CITED2 construct, residues 204–269, binds to TAZ1 in a similar manner, as manifested by changes in the backbone amide peaks of $^{15}$N-labeled TAZ1 (data not shown), but has a tendency to precipitate upon complex formation. The shorter CITED2 construct (residues 220–269) showed high affinity to TAZ1 ($K_d = 13$ nM, Table I) with less tendency to precipitate upon binding to TAZ1 and was therefore used for structure determination. Binding is in slow exchange on the chemical shift time scale, as evidenced by the appearance of new CITED2 peaks in the bound form and the disappearance of free CITED2 peaks when $^{15}$N-labeled CITED2 is added to unlabeled TAZ1.

The changes in chemical shifts for the CITED2 protein upon binding indicate the formation of a helical structure in the N terminus, with an extended structure in the center of the sequence. This is in contrast to the observation for HIF-1α of the formation of helical structure in several locations throughout the sequence. Measurement of the heteronuclear $^1$H-$^1$N NOE for CITED2 in the complex (Fig. 3) shows that the carboxy-terminal 10 residues are relatively mobile. As observed for the binding of HIF-1α to TAZ1 (13), a number of significant shifts in HSQC cross-peak positions were observed when unlabelled CITED2 was added to $^{15}$N-labeled TAZ1. Chemical shift mapping (Fig. 4) indicated that different regions of TAZ1 are perturbed by HIF-1α and CITED2, suggesting that the complex structures of the HIF-1α and CITED2 are likely to be different.

Table I shows the binding constants of TAZ1 with CITED2 and two HIF-1α constructs (residues 776–826 and 790–826)
obtained by ITC. The two HIF-1α constructs were used for binding studies to correlate the measured affinities with structural results. The longer HIF-1α (residues 776–826) was used in the CBP complex (20), and the shorter HIF-1α (residues 790–826) was used in the p300 complex (13). The structure of the CBP TAZ1-HIF-1α (residues 776–826) showed intermolecular interactions involving the amino-terminal extension of HIF-1α, which likely contribute to the tighter binding affinity of the longer HIF-1α (residues 776–826) compared with that of the shorter HIF-1α (residues 790–826). ITC results indicate that CITED2 binds to TAZ1 with comparable affinity to the longer HIF-1α construct, but it binds approximately four times more tightly than the shorter HIF-1α construct.

Three-dimensional structures for the TAZ1-CITED2 complex were calculated using the program DYANA (29) and refined by molecular dynamics and simulated annealing with the program AMBER (30), using intramolecular and intermolecular NOEs and dihedral angle constraints as listed in Table II. A superposition of the 20 lowest energy structures of the TAZ1-CITED2 complex is shown in Fig. 5A, and a single member of the ensemble is shown in Fig. 5B. As was observed in the HIF-1α complex and in free TAZ1 (2), TAZ1 adopts a compact fold containing predominantly helical secondary structure stabilized by three zinc atoms. Upon complex formation, the activation domain of CITED2 wraps around TAZ1, and its folded structure is maintained by numerous intermolecular hydrophobic contacts. The structure of CITED2 is defined primarily by its association with TAZ1, as indicated by the much higher number of intermolecular NOEs (299; Table II) compared with only 13 intramolecular long-range NOEs (i – j > 4) for CITED2 in the complex.

The structure of the TAZ1 domain in the complex with CITED2 is very similar to that in the HIF-1α complex (13, 14), consisting of four helices, namely α1 (Pro-347 to Ala-372), α2 (His-383 to His-396), α3 (Ala-406 to Cys-421), and α4 (Cys-429 to Asn-438), and three HCC/C-type zinc-binding motifs, i.e. Zinc1 (His-362, Cys-366, Cys-375, and Cys-384), Zinc2 (His-393, Cys-397, Cys-405, and Cys-408), and Zinc3 (His-417, Cys-421, Cys-426, and Cys-429). Each of the zinc-binding sites contains one histidine and three cysteine residues distributed across the ends of two helices and joined by a flexible loop. The two helices that form each zinc-binding site are packed at an angle against each other. The four helices are then packed against each other via hydrophobic interactions to enclose a hydrophobic core in the center of the molecule. Intermolecular interactions at the end of TAZ1 α4 and CITED2 (between residues Ala-435 and Lys-438 of TAZ1 and Val-227 of CITED2) stabilize the end of α4, making it significantly longer than the α4 helix in the TAZ1-HIF-1α (5 residues) (13) and p300 TAZ1-CITED2 complexes (5 residues) (20). In the present complex, α4 forms a regular α-helix from Ala-435 to Lys-438.

The structured portion of CITED2 in the complex begins at Phe-222, forming an extended structure with hydrophobic contacts with TAZ1 α1 and α4 (Fig. 6A). The aromatic ring of Phe-222 binds in a hydrophobic pocket formed by the TAZ1 α1 residues Leu-361, His-364, and Lys-365, whereas Ile-223 fits in the hydrophobic interface formed by α1 (Leu-361 and Lys-365) and α4 (Leu-432). The polar side chains of the highly conserved Asp-224 and Glu-226 residues are directed away from the interface and are close to the Lys-365, Lys-437, and Arg-438 of TAZ1. A well defined helix, α4, from Glu-225 to Met-235, fits in a groove formed by the TAZ1 α1/α4 interface and is stabilized by an extensive network of intermolecular hydrophobic contacts (Fig. 6A). Highly conserved nonpolar residues on α4 are in direct hydrophobic contact with TAZ1 α1 and α4. Val-227 interacts with α1 residues Ala-435, Ser-436, and Lys-438, whereas Leu-228 is in a hydrophobic pocket formed by α1 residues Val-358, Leu-361, His-362, and Lys-365 and α4 residue Leu-432. The hydrophobic side chain of Met-229 fits in a cleft formed by the aromatic ring of His-362 and the methyl groups of Leu-381. The invariant Leu-231 interacts with α1 (Val-358) and α4 (Pro-431, Leu-432, and Ala-435) residues. At the end of α4, Val-232 interacts mainly with Val-358 and Leu-381, Ile-233 interacts with Leu-381, and Met-235 interacts with α1 residues Lys-351, Gln-354, and Val-358. The polar residues Glu-225, Glu-226, Ser-230, and Glu-234 are all located on the solvent-exposed face of α4.

The axis of CITED2 helix α4 packs at angles of 27 and 59° against the TAZ1 α1 and α4 helices, respectively. Binding of CITED2 α4 into the TAZ1 α1/α4 interface does not increase the separation between α1 and α4, nor does it change the relative α1/α4 orientation. The structure of TAZ1 in complex with HIF-1α (13, 14), as well as the structure of free TAZ1 (2) show very similar distances and helix orientations between α1 and α4, suggesting that the TAZ1 α1/α4 interface presents a hydrophobic groove tailor-made for protein-protein interaction.

Following α4, CITED2 forms a loop from Gly-236 to Glu-242 that binds in a cleft of TAZ1 formed between α1 and a loop of the first zinc-binding site (Zn1) near the N terminus of α2. There are two highly conserved nonpolar residues in this loop, Leu-237 and Ile-240, that pack against hydrophobic residues in TAZ1 (Leu-237 with Gln-355, Val-358, and Leu-359 in α1 and Ile-240 with Leu-381 in the Zn1 site) and also participate in intramolecular hydrophobic interactions (Ile-240 with Leu-237 and Leu-243) (Fig. 6A). The polar residues Asp-238, Arg-239, Lys-241, and Glu-242 are all solvent-exposed and do not participate in protein binding to TAZ1. Gly-236 is invariant and populates the α2 region of the Ramachandran plot, an indication that this residue participates in a carbonyl-terminal helix cap for α4. This loop also contains Glu-242, which has a significantly more flexible backbone than neighboring residues, as indicated by the dip in [3H]-15N heteronuclear NOE data (Fig. 3). The increased flexibility at Glu-242 may indicate that this loop functions as a hinge between the more rigid α4 helix and the LPEL motif described below.

Toward the middle of the sequence, the activation domain of CITED2 contains a sequence of four residues, LPEL, residues...
243–246, that binds tightly in the TAZ1 α3/α2/α1 junction (Fig. 6B). The side chain of CITED2 Leu-243 packs tightly in a hydrophobic pocket lined by the TAZ1 residues Leu-352, Glu-356, Leu-359, the α3 residues Thr-386, Met-387, Val-390, and the α1 His-407 aromatic ring. CITED2 Pro-244 interacts mainly with the TAZ1 Leu-352 in α3 and, although Glu-245 is solvent-exposed, it shows intermolecular NOEs to α2 residues Ala-406 and His-407. Leu-246 packs in a hydrophobic cavity at the α1/α2 interface formed by the α1 residues Lys-349, Leu-352, and Ile-353 and the α2 residues Ser-410 and Ile-414. The homologous sequence in the HIF family is LP(Q/E)L, residues 792–795, where Q/E indicates either glutamine (HIF-1α) or glutamate (HIF-2α) at position 794. A similar network of intermolecular contacts was also observed for the corresponding HIF-1α LPQL (residues 792–795) region in the TAZ1-HIF-1α complex (13, 14), and even the side chains of these residues occupy similar positions in the two complexes (Fig. 7B). The LPEL loop is well defined in the structures, as indicated by the backbone root mean square deviation to the mean structure of 0.60 Å and the backbone heteronuclear [1H]-15N NOE, comparable with that of the well structured α3 helix (Fig. 3). This is likely due to the extensive network of intermolecular hydrophobic contacts formed by the CITED2 LPEL. Although the LP(E/Q)L loop is similar in both CITED2 and HIF-1α, other TAZ1-binding proteins in general do not contain this motif. These data suggest that the LP(E/Q)L loop might be a unique feature of HIF-1α or CITED2 interactions with the TAZ1 domain.

Immediated the following the LPEL loop, the aromatic ring of Trp-247 interacts with residues of the Zn2 binding site and the α3 helix (Gln-404, Ala-406, and Ala-409). Leu-248 participates in intramolecular (with Ile-353 in α1 and Ile-414 in α2) and intermolecular (with Leu-246, Phe-253, and Phe-255) hydrophobic interactions. There is a poorly defined loop from Gly-249 to Glu-252 with solvent-exposed side chains and generally weak intermolecular contacts. The intramolecular and intermolecular hydrophobic contacts formed by Leu-248, Phe-253, Phe-255, Met-256, and Thr-257 define the interaction of CITED2 at the α3/α2 interface, shown in Fig. 6C. The Phe-253 aromatic ring packs in a pocket formed by Lys-349, Arg-350, Ile-353, and Ile-414. The aromatic ring of Phe-255 participates in hydrophobic interaction with α2 (Ala-345 and Ile-353), α3 (Ile-414 and His-407), and α1 (Val-390) residues. The side chain of Met-256 occupies a hydrophobic pocket formed by α1 (Ala-345, Arg-350, and Ile-353), α2 (Ile-414), and α3 (Pro-427 and Val-428) residues. Finally, Thr-257 shows intermolecular contacts with Thr-344 and Ala-345 in α1. Weak intermolecular NOEs from Asp-258, Phe-259, and Val-260 in the CITED2 flexible tail to the α1 residues Pro-343, Thr-344, and Ala-345 probably reflect transient contacts, because the last 10 carboxyl-terminal residues of CITED2 are apparently unstructured, as indicated by diminished [1H]-15N heteronuclear NOE values (Fig. 3).

**DISCUSSION**

With the recent publication of the solution structure of the complex of CITED2 with the TAZ1 domain from a homologous protein, p300 (20), there are now four similar structures, including the two HIF-1α complexes (13, 14), that can be compared to determine the nature of the competition between HIF-1α and CITED2 for binding to TAZ1. A superposition of the TAZ1 structures in the two CBP complexes (Fig. 7A) shows clearly that the HIF-1α and CITED2 domains bind to the same general area on one side of the TAZ1 domain, but with quite
different structures except in the immediate vicinity of the LP/E/QL sequence. Where CITED2 forms a well structured helix α4 that binds between α1 and α4 of TAZ1, the HIF-1α polypeptide is poorly structured, though with helical backbone dihedral angles. This part of the HIF-1α sequence was omitted from the construct used to determine the structure of the p300-HIF-1α complex (14), with significant effects on the dissociation constant for the complex. Table I shows that the removal of 14 residues at the amino terminus of the HIF-1α construct results in an ~5-fold decrease in the binding affinity. The TAZ1 affinity of the longer HIF-1α (residues 776–826) construct used in the structure determination of the CBP complex (13) is comparable with that of CITED2 (Table I). A major part of the HIF-1α binding surface consists of the αc helix on the backside of the molecule as viewed in Fig. 7A. CITED2 does not contact TAZ1 at all in this region. Thus, it appears that the two activation domains, HIF-1α and CITED2, bind to partially overlapping surfaces of TAZ1 with similar affinities but significantly different structures.

Comparison of the CBP and p300 TAZ1-CITED2 Complexes—Freedman et al. (20) have recently reported the structure of the p300 TAZ1 domain bound to a short fragment of CITED2 (residues 216–259). The CBP TAZ1 and p300 TAZ1 domains share ~92% sequence identity, and most of the differences are in loop regions where they do not significantly alter the structure of the domain. One exception is in the carboxyl terminus of TAZ1, where we observed a longer helix α4 in the TAZ1-CITED2 complex. The presence of a helix-destabilizing glycine residue at the end of the p300 TAZ1 α4 probably discourages longer helix formation in this protein.

The binding of CITED2 to the CBP TAZ1 and p300 TAZ1 domains shows slight differences. Although for the most part the structures of CITED2 in the complex are very similar indeed, the p300-CITED2 complex shows the presence of a 3₁₀ helical region at the C terminus of CITED2 (residues 254–256) (20). We do not see such a helix in the CBP-CITED2 complex, and the chemical shift index indicates no helix in this region. Different CITED2 constructs were used in the two complexes. The construct used for the CBP complex (residues 220–269) is significantly longer at the carboxyl terminus than the one used in the p300 complex (residues 216–259). The additional 10 residues at the carboxyl terminus appear to be highly flexible according to ¹⁵N relaxation data (Fig. 3) and do not appear from the NOESY spectrum to make contact with the TAZ1 domain.

Differences in the lengths of constructs used to form the complexes can influence conclusions based on affinity measurements and structures. For example, Freedman et al. (20) conclude that CITED2 shows a higher affinity for p300 TAZ1 compared with HIF-1α, whereas we report here that CITED2 and HIF-1α show very similar affinities to the CBP TAZ1 domain (Table I). This discrepancy is probably due to the difference in constructs used. Table I also shows measured $K_d$ values for constructs of HIF-1α of various lengths. The construct used for the structure determination of the HIF-1α-TAZ1 complex of CBP by Dames et al. (13) included residues 776–826, whereas that used by Freedman et al. (14) for the complex with TAZ1 of p300 included residues 786–826. The $K_d$ values for these two constructs differ by a factor of 5 (Table I), an indication that the amino-terminal residues of HIF-1α do contribute to the binding interaction.

Structural Basis of HIF-1α Inhibition by CITED2—Comparison of the structures of the complexes of the TAZ1-HIF-1α (13) and TAZ1-CITED2 complexes provides insights into how CITED2 inhibits HIF-1α by displacing it from TAZ1. One major difference between CITED2 and HIF-1α is in the tight binding of the CITED2 αa helix to the interface formed by TAZ1 αa and αc. The αa helix of CITED2 forms intimate interactions with TAZ1 that result in a high degree of restriction of the backbone motions (Fig. 3). In contrast, the αa helix of HIF-1α (13) appears to interact much more weakly with the same region of TAZ1. This is shown by the paucity of intermolecular NOEIs (13) and the increased conformational flexibility of the backbone, as indicated by ¹⁵N relaxation data.³ Chemical shift mapping of the interaction sites of HIF-1α and CITED2 on TAZ1 (Fig. 4) also indicates that the backbone amide resonances of the αc helix residues are perturbed by HIF-1α to a lesser degree than by CITED2.

Fig. 4 shows that binding to CITED2 resulted in large chemical shift changes in TAZ1 residues α₁, α₂, and α₄. The large chemical shift changes in TAZ1 α₁ and α₂ helices are due to the tight binding interaction of CITED2 helix α₄. The CITED2 residues Leu-237 and Ile-240, located before the LP/E/L sequence, also bound tightly near the TAZ1 α₄ helix, resulting in large chemical shift changes seen in His-383, Thr-386, and Met-387 in Fig. 4. In HIF-1α, the corresponding residues that

³ J. C. Lansing and P. E. Wright, unpublished data.
are located before the LPQL loops do not make intermolecular contacts with TAZ1 and thus do not perturb the backbone chemical shifts as much as those seen in the CITED2 complex.

Another difference between the CITED2 and HIF-1α complexes is that the packing of αC of CITED2 with helix α4 of TAZ1 stabilizes the end of αA, making it longer than in the TAZ1-HIF-1α complex. Also, CITED2 does not have a helix equivalent to HIF-1α αC, which interacts strongly with TAZ1 αA, because the carboxyl-terminal 10 residues of CITED2 are disordered and, at best, make only transient contact with TAZ1. The absence of an equivalent αC helix is also reflected in the smaller buried surface area of CITED2 (1900 Å²) compared with that of HIF-1α (2400 Å²). The buried surface area in the CITED2 complex is more similar to that in the complex with the truncated HIF-1α construct (residues 790–826), which lacks the HIF-1α helix αA (1800 Å²).

Hydroxylation of Asn-803 in HIF-1α inhibits transcriptional activity by disrupting the TAZ1-HIF-1α interaction (12). Asn-803 is part of the HIF-1α αA helix that binds in the TAZ1 α4/α3 interface (13, 14). The CITED2 residues Phe-253, Phe-255, and Met-256 form hydrophobic contacts with TAZ1 α4/α3 residues at the site occupied by HIF-1α αA. That the TAZ1 α4/α3 interface is able to participate in hydrophobic interaction is consistent with the observation that an alanine point mutant at position 803 of HIF-1α showed tight binding to p300 and did not repress the HIF-1α transcriptional activity (12).

How Do CITED2 and HIF-1α Displace One Another?—The observation that HIF-1α activates the transcription of CITED2 during the hypoxic response and that CITED2 inhibits the transcriptional activity of HIF-1α (16) suggests feedback loop regulation. Based on the NMR structures of TAZ1-CITED2 and TAZ1-HIF-1α, we propose the following hypothesis on the mechanism by which CITED2 displaces HIF-1α from TAZ1 and vice versa. Both the HIF-1α and CITED2 binding domains appear to be unstructured in the free state. The flexibility and weak binding of HIF-1α αA to TAZ1 α3/α4, plus the fact that the CITED2 αA binds tightly in the same position, suggest that CITED2 displaces HIF-1α by binding tightly to the TAZ1 α3/α4 region first and then competes for binding of the HIF-1α LPQL loop with its own LPQL motif, finally displacing the whole HIF-1α from TAZ1. When the level of HIF-1α increases, it begins to displace CITED2 from TAZ1 by a tight binding of the HIF-1α αC to the side of TAZ1 α3/α4 not used by CITED2. Indeed, a peptide that included the HIF-1α helix αC sequence (HIF-1α residues 808–826) showed binding to TAZ1 (Kd = 500 μM⁻¹) as determined by tryptophan fluorescence anisotropy. In this model, hydroxylation of Asn-803, which represses the transcriptional activity of HIF-1α, is viewed as decreasing the affinity of HIF-1α to TAZ1 but would not completely prevent the other parts of HIF-1α from binding to TAZ1. Thus, displacement could be viewed as a combination of the “mass action” effect of a disparity of concentration, combined with the kinetic effect of competition between two ligands with similar affinity for a similar, but not identical, binding site. The switch between binding of one ligand and the other could be accomplished rapidly, because there is no need for complete dissociation.
ation of one complex before the competing molecule can bind and displace it.

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