Dynamics of the Hypoxia-inducible Factor-1-Vascular Endothelial Growth Factor Promoter Complex

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Some transactivator-promoter complexes are highly dynamic due to active disruption of the complex by proteolytic or non-proteolytic mechanisms, and this appears to be an important mechanism by which their activity is governed tightly and eventually terminated. However, the generality of these mechanisms is unclear. In this report, we address the dynamics of hypoxia-inducible factor-1 (HIF-1) binding to the vascular endothelial growth factor promoter. HIF-1 is a heterodimeric transcription factor whose activity is triggered by an increase in HIF-1α levels in hypoxic cells. A “competition ChIP” assay is employed to demonstrate that HIF-1α forms a kinetically stable complex with the native vascular endothelial growth factor promoter that has a half-life in excess of 1 h. Thus, HIF-1 activity does not require rapid proteolytic turnover of the promoter-bound transactivator, nor is the activator-promoter complex constantly disassembled by chaperones. However, we do find that after cessation of the inducing signal, HIF-1 activity is slowly returned to basal levels by proteasome-mediated proteolysis of the promoter-bound HIF-1α protein.

The former set of events represents one of the several intimate connections between the ubiquitin-proteasome pathway and RNA polymerase II transcription. Indeed, in several cases, proteasome function has been found to be essential for the full activity of particular activators, and, in a few cases, the available data argue that this reflects a requirement for the periodic clearance of polyubiquitylated activators from the promoter (9, 10, 15, 16), although activator function and turnover can be uncoupled by mutation (17) in the case of the estrogen receptor. In addition to activator turnover, there have been reports of proteasome-mediated turnover of other promoter-bound transcription factors as well, including coactivators and general transcription factors (18, 19). These findings and others have resulted in the proposal of the so-called “timer” (6) and “black widow” (3) models, which posit that activators and other transcription factors are polyubiquitylated and subsequently destroyed by the proteasome as a consequence of driving transcription. Since it is also the case that many activators must be monoubiquitylated in order to function efficiently (20–23), this provides an appealing mechanism for coupling the licensing of activator function to its imminent destruction by extension of the ubiquitin chain from one to more than four Lys48-linked residues (24, 25) and thus requiring continued signaling and fresh activator to maintain gene transcription over time.

The detailed studies required to probe the dynamics of transcription factor-promoter interactions have been done in only a handful of cases, so the generality of either proteolytic or nonproteolytic destabilization of activator-promoter complexes during activated gene expression is unclear. Some exceptions have already been noted. *Drosophila* heat shock factor binds to native promoters in polytene chromosomes to form kinetically stable complexes that do not cycle rapidly by either a proteolytic or nonproteolytic mechanism (26). Indeed, it was already clear that proteasome-mediated cycling was not required for heat shock factor activity, since proteasome inhibitors stimulate heat shock gene expression (27, 28). In yeast, evidence has been presented for strong coupling between proteasome-mediated turnover and transactivation by Gcn4 (10). In the case of the paradigmatic transactivator Gal4, we have reported that Gal4-GAL promoter complexes are stable under inducing conditions but are kinetically labile under noninducing conditions and have presented other evidence consistent with the idea that Gal4 must be in the act of transactivation in order to form kinetically stable complexes (29). However, this conclusion has proven controversial, and circumstantial evidence in favor of proteolytic turnover of active Gal4 has been presented by others (10, 30).
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Outside of these studies in lower eukaryotes and the above referenced work on a few nuclear hormone receptors, little is known about the dynamics of transactivator-promoter complexes, particularly in mammalian cells. To begin to address this issue, we probe here the dynamics of hypoxia-inducible factor-1 (HIF-1) binding to the VEGF promoter in HeLa cells. HIF-1 is an important part of the oxygen-sensing system, playing critical roles in both embryonic development and postnatal physiology in all metazoans (31, 32). HIF-1 is a heterodimeric transcription factor composed of HIF-1α and HIF-1β (also known as ARNT) subunits. HIF-1β is expressed constitutively, whereas the level of HIF-1α is regulated tightly. Under normoxic conditions, iron- and oxygen-dependent prolyl hydroxylases (33) modify two proline residues in HIF-1α, rendering the protein a substrate for the VHL-containing E3 ubiquitin ligase complex (34). This triggers proteasome-mediated degradation of HIF-1α, keeping its basal level very low. However, under conditions where the hydroxylases are less active, such as hypoxia or in the presence of certain inhibitory metal ions, such as Co2+ (35), HIF-1α is not hydroxylated and polyubiquitylated efficiently, leading to a dramatic rise in its level. As it accumulates, HIF-1α associates with HIF-1β protein, and the heterodimer binds to hypoxia-responsive elements (HREs) in various promoters and drives expression of the corresponding genes. HIF-1α-mediated transcription has been a focus of intense study in the cancer community, since its activity is critical for angiogenesis and hence the growth of solid tumors (32).

We show here that HIF-1 forms a kinetically stable complex with HREs in the VEGF promoter in HeLa cells under inducing conditions that has a lifetime of well over 1 h. This rules out either proteolytic or nonproteolytic cycling of the activator on this promoter, at least under the conditions studied here. Once the inducing signal is removed, we find that HIF-1-promoter complexes decay slowly by a mechanism that does require proteasome-mediated proteolysis and appears to involve attack of the proteasome on the DNA-bound activator.

EXPERIMENTAL PROCEDURES

See supplemental material for plasmid construction information and for sources of the chemicals.

Cell Culture—HeLa cells or COS7 cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen). For experiments using constructs containing estrogen receptor (ER) LBD, the cells were grown in phenol red-free Dulbecco’s modified Eagle’s medium supplemented with 10% charcoal-treated fetal bovine serum. Transfections were performed using Lipofectamine 2000 (Invitrogen) or TransIT-L1T1 (Mirus).

Antibodies, Western Blotting, and Immunoprecipitation—The following antibodies were used to detect protein expression by Western blot: HIF-1α (catalog number 07-628; Upstate Biotechnology, Inc.), FLAG M2 antibody (Sigma), ARNT (HIF-1β) (catalog number H-172; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For immunoprecipitations (IPs), protein complexes were precipitated with anti-FLAG M2-Sepharose beads (Sigma). Precipitated proteins were subjected to Western blotting with anti-ARNT (catalog number H-172).

RNA Analysis—Cells were collected, and RNA was purified by the RNeasy kit (Qiagen). RNA was then converted to cDNA using the Stratascript first strand synthesis system (Stratagene) and oligo(dT) primers. cDNA samples were analyzed by real time PCR (iCycler Thermal Cycler; Bio-Rad) with primers that amplify the corresponding mRNAs. The signals were normalized to tubulin mRNA levels.

Chromatin Immunoprecipitation (ChIP) and Real Time PCR Quantitation—The antibodies or the antibody conjugates used in the ChIP assays are as follows: anti-HIF-1α (rabbit polyclonal, Upstate Biotechnology catalog number 07-628), anti-FLAG M2 antibody conjugate (mouse monoclonal; Sigma catalog number A2220), anti-HA antibody conjugate (mouse monoclonal HA-7; Sigma catalog number A2095), anti-β1 subunit of the 20 S proteasome subcomplex, and anti-Rpt4 of the 19 S proteasome subcomplex (from Dr. Devanjan Sikder, University of Texas Southwestern Medical Center).

ChIP assay was performed essentially by following the protocol of Upstate Biotechnology with minor modifications. A detailed ChIP protocol, the primer sequences for PCR amplification, and the real time PCR quantitation procedure can be found in the supplemental materials.

RESULTS

A “Competition ChIP” Assay for the Analysis of Native HRE-HIF-1 Complexes—We recently introduced a novel technique called the “competition ChIP” assay that allows the rate of dissociation of yeast transcription factors, such as Gal4 from native promoters, to be monitored in vivo. Here we adapt this assay to mammalian cells and the somewhat more complex situation of the HIF-1 heterodimer. Fig. 1 shows a schematic of the assay. HeLa cells were transfected with a plasmid that expresses a fusion protein linking FLAG-tagged HIF-1α DNA-binding and HIF-1β interaction domains (residues 1–390), the glucocorticoid receptor ligand-binding domain (GR LBD; residues 499–777), and enhanced green fluorescent protein. This protein, hereafter called FLAG-HLG, was expressed at high levels under the control of the strong cytomegalovirus enhancer and promoter. In the absence of steroid ligand, FLAG-HLG is held in an inactive state by a high affinity association with Hsp90 and is thus unable to access HREs in the HeLa genome. After the addition of a steroid agonist or antagonist, the FLAG-HLG-Hsp90 complex is disrupted, allowing the fusion protein access to HREs in the nucleus. When HIF-1α levels are high, these sites will already be occupied by native HIF-1 heterodimers, and the fusion protein will be able to associate only upon dissociation of the native transactivator from the promoter. If the fusion protein is present in large excess over the native HIF-1α, this renders dissociation of the latter essentially irreversible. Thus, by monitoring the dissociation of the native HIF-1α protein and the association of the FLAG-HLG compet-

2 The abbreviations used are: HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor; E3, ubiquitin-protein isopeptide ligase; HRE, hypoxia-responsive element; IP, immunoprecipitation; ChIP, chromatin immunoprecipitation; HA, hemagglutinin; GR, glucocorticoid receptor; LBD, ligand-binding domain; ER, estrogen receptor; FRAP, fluorescence recovery after photobleaching; DBD, DNA-binding protein; qPCR, quantitative PCR.
In order to ensure that the scenario shown schematically in Fig. 1 is operative in our assays, several important criteria must be met. First, it is important that the competitor protein indeed be present at much higher levels than the native factor. Otherwise, reassociation of the latter would complicate interpretation of the data. Although the levels of native HIF-1α and FLAG-HLG could not be compared directly due to the lack of a good antibody that recognizes epitopes in both, we employed appropriate internal standards in quantitative Western blotting experiments to effect an indirect comparison. These data showed that the amount of FLAG-HLG protein is at least 10-fold higher than that of HIF-1α, even under conditions where levels of the native factor are stimulated by treatment with CoCl$_2$, a commonly used hypoxia mimetic that acts by inhibiting the activity of the iron-dependent hydroxylases that target HIF-1α (35) (supplemental Fig. S1).

Another assumption in the assay is that the addition of the steroid that triggers release of the competitor protein has no effect on binding of HIF-1 to promoters. To address this, ChIP assays were conducted in the presence and absence of dexamethasone and RU-486. The results showed no appreciable effect of these two small molecules on the HIF-1-VEGF promoter association (supplemental Fig. S2).

Also important is that the addition of steroid to the FLAG-HLG-expressing cells effects release of active competitor protein rapidly and that it associates efficiently with HIF-1β. As shown in Fig. 2A, when an extract prepared from dexamethasone-treated HeLa cells expressing FLAG-HLG was exposed to immobilized anti-FLAG antibody, HIF-1β was observed to co-immunoprecipitate. The HIF-1β band was not observed in cells

**FIGURE 2. Validation of the activity of the FLAG-HLG fusion protein.**

A, binding of FLAG-HLG to the HIF-1β protein. Whole cell lysates from HeLa cells that were or were not transfected with a plasmid expressing the FLAG-HLG fusion protein were immunoprecipitated with beads containing anti-FLAG antibody, and the precipitates were probed for the presence of the HIF-1β by Western blotting. B, transactivation function of FLAG-HLG. HeLa cells co-transfected with plasmids expressing the FLAG-HLG construct and containing an HRE-Luc reporter plasmid were treated with the indicated concentrations of dexamethasone or RU486. Luciferase levels were measured 40 h later. C, transactivation activity of FLAG-HLG on the native VEGF gene. HeLa cells transfected with the FLAG-HLG expression plasmid were treated with dexamethasone (100 nM) or RU486 (100 nM) for 2 h. VEGF mRNA levels were detected by agarose gel electrophoresis after PCR and quantified by qPCR as well. D, time course of binding of FLAG-HLG to the VEGF promoter after induction with dexamethasone, as monitored by ChIP using anti-FLAG antibody. The immunoenriched DNA samples were detected by agrose gel electrophoresis after PCR (see gels) and quantified by qPCR as well (see graph).
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lacking FLAG-HLG. It is also noteworthy that only a fraction of the HIF-1β present in the extract was associated with the FLAG-HLG (compare input with IP), indicating that, as expected, there is a large pool of excess available HIF-1β. This is important for this study, because it suggests that the FLAG-HLG competitor protein need not compete with endogenous HIF-1α for limiting HIF-1β.

To assess if the fusion protein is functional as a transcriptional activator, the expression of genes targeted by HIF-1 was measured before and after the addition of steroid. The GR LBD contains a ligand-dependent transactivation domain called AF-2 that recruits coactivators upon binding a steroid agonist, such as dexamethasone. Therefore, as expected, treatment of cells expressing FLAG-HLG with dexamethasone resulted in a strong increase in the expression of a HIF-1-responsive luciferase reporter gene (Fig. 2B) as well as the native VEGF gene (Fig. 2C). When the cells were exposed to the GR antagonist RU486 (mifepristone), the level of stimulation of these genes was much lower, as expected (Fig. 2, B and C). We conclude that FLAG-HLG does indeed associate with HIF-1β and HREs in the presence of steroid and thus can act as a functional, inducible mimic of HIF-1α.

Finally, it was important to deduce the amount of time necessary for the FLAG-HLG competitor protein to be released from Hsp90, move to the nucleus, and occupy HREs, since this defines the minimal time resolution of the assay. Cells that expressed FLAG-HLG but did not express significant levels of native HIF-1α (i.e. normoxic conditions) were treated with dexamethasone, and a ChIP assay using anti-FLAG antibody was employed to monitor FLAG-HLG association with the VEGF promoter over the following 1 hr. Under these conditions, the FLAG-HLG protein does not have to compete with native HIF-1α for binding to the promoter, and its rate of association therefore measures the amount of time required for a buildup of free, functional protein in the nucleus after steroid addition. As shown in Fig. 2D, saturation of the HREs occurred in about 10 min with an apparent t½ of about 5 min. This defines the minimal time resolution of this technique.

We conclude that all of the criteria necessary for applying this assay to the study of HRE-HIF-1 complexes have been met and that this technique should be suitable for monitoring the half-life of native HRE-HIF-1 complexes.

**HRE-HIF-1 Complexes Are Kinetically Stable under Inducing Conditions**—As expected, when HeLa cells were treated with CoCl2, thus inhibiting hydroxylation of HIF-1α, a strong induction of HIF-1α levels and HIF-1 activity was observed (supplemental Fig. S3). Accordingly, a strong ChIP signal due to binding of native HIF-1α to the HREs in the VEGF promoter resulted, which peaked 3–5 h after treatment with CoCl2 (Fig. 3A). To determine the kinetic stability of this complex, we employed the competition ChIP assay. 40 h after transfection of the cells with the FLAG-HLG expression vector, they were treated with CoCl2 for 5 h to fully induce HIF-1 activity. Dexamethasone was added at this point (called time 0) to initiate the competition between native HIF-1α and FLAG-HLG. ChIP assays using an antibody that recognizes native HIF-1α but not FLAG-HLG or using anti-FLAG antibody, which does not recognize native HIF-1α (see supplemental Fig. S1), were then used to monitor association of each protein with the promoter over time. As shown in Fig. 3B, the ChIP signal due to native HIF-1α displayed little or no loss in intensity for at least 60 min after the addition of dexamethasone. Correspondingly, little or no signal indicative of association of FLAG-HLG with the promoter could be detected, despite the large excess of this protein over the native HIF-1α.

![FIGURE 3. Assessment of the kinetic stability of the HIF-1-VEGF promoter complex using the competition ChIP assay.](image-url)
The same result was obtained when the competitor protein contained ERα LBD in place of the GR LBD, and the experiment was triggered by the addition of estradiol (supplemental Fig. S4), demonstrating that the nature of the ligand-binding domain and the steroid did not affect the result.

To ensure that the FLAG-HLG protein is indeed capable of competing successfully with native HIF-1α for HRE binding, cells were treated with CoCl2 and dexamethasone simultaneously. Since FLAG-HLG activity is induced much more quickly than the time required for significant native HIF-1α accumulation, this sets up a situation in which FLAG-HLG is able to occupy the promoter first. The question then becomes whether it is able to remain stably associated once native HIF-1α levels build up. As shown by ChIP analysis (supplemental Fig. S5), the fusion protein under these conditions clearly associated with the promoters, whereas the native HIF-1α was largely occluded from the promoter. We conclude that the stable binding of HIF-1α to the HRE in the presence of excess competitor protein (Fig. 3B) cannot be explained by inefficient binding of FLAG-HLG compared with native HIF-1α.

To rule out the possibility that the inability of FLAG-HLG to associate with the promoters already occupied by HIF-1α is due to limited HIF-1β, HIF-1β protein was overexpressed along with the competitor fusion protein, and the competition ChIP experiment was repeated. The same result was observed: the native HRE-HIF-1 complex was extremely stable (supplemental Fig. S6).

To check the stability of the HRE-HIF-1 complex under bona fide hypoxic conditions, after transfection of the FLAG-HLG vector, HeLa cells were incubated in 1% O2 for 6 h to induce hypoxia. The competition ChIP assay was then repeated, and the same result was observed (supplemental Fig. S7). Again, native HIF-1α remained stably associated with the promoter, and little FLAG-HLG promoter binding was detected up to 1 h after the addition of the steroid.

From the above experiments, we conclude that HIF-1 indeed forms a kinetically stable complex with HREs that is insensitive to competition with excess protein for well over 1 h. This result argues against the idea that rapid turnover of promoter-bound HIF-1α is important for high activation potency.

The Presence of a Functional Activation Domain Is Essential for Sustaining Stable Association with Promoters—In our previous study of Gal4-promoter interactions in vivo, we found that the kinetic stability of the activator-DNA complex was highly dependent on the presence of a functional activation domain (29). To determine if regions other than the DNA-binding domain in HIF-1α are required for its stable association with HREs in HeLa cells, an HA-tagged HIF-1α DNA-binding domain (DBD) construct was created, and the dynamics of the HA-HIF-1α DBD-promoter complex was studied. First, to make sure the protein is functional, HeLa cells were transfected with this construct, and the effect of the protein on the transcription events driven by the native HIF-1α proteins and the FLAG-HLG fusion proteins was monitored. In other words, if the HIF-1α DBD alone is competent to associate with HREs in cells, this activation domain-lacking polypeptide should act as an inhibitor of HIF-1α-activated transcription when present at high levels. As expected, expression of the HA-HIF-1α DBD protein inhibited the transcription of a HIF-1α-responsive reporter gene activated by both native HIF-1 and by the FLAG-HLG protein (Fig. 4A and B).

To determine the kinetic stability of HRE complexes containing only the HIF-1α DBD, HeLa cells were co-transfected with the plasmids encoding the HA-HIF-1α DBD and FLAG-HLG in a mass ratio of 1:10, in order to artificially set up a situation in which the FLAG-HLG protein is in excess. ChIP analysis using anti-HA antibody and anti-FLAG antibodies was used to monitor promoter occupancy of the HA-HIF-1α DBD protein and the FLAG-HLG protein, respectively. The immunoenriched DNA samples were detected by agarose gel electrophoresis after PCR (see gels) and quantified by qPCR as well (see graph; the mean of three experiments is shown).

FIGURE 4. Role of the activation domain in the formation of a kinetically stable HIF-1α-promoter complex. A, assessment of the DNA-binding activity of the HIF-1α DBD. HeLa cells either mock-transfected or transfected with a plasmid expressing HA-HIF-1α DBD were treated with CoCl2 and the HRE-responsive luciferase levels were compared. HA-HIF-1α DBD interfered with HIF-1α-mediated transcription, indicating that it is capable of binding to DNA. B, the HA-HIF-1α DBD protein inhibits the activation potential of the FLAG-HLG protein. HeLa cells were co-transfected with the FLAG-HLG and the HA-HIF-1α DBD constructs at a mass ratio of 1:1 and treated with different concentrations of dexamethasone. The luciferase induction levels were compared with those from HeLa cells transfected with FLAG-HLG construct alone. C, assessment of the kinetic stability of the HA-HIF-1α DBD-promoter complex by competition ChIP assay. HeLa cells co-transfected with the FLAG-HLG- and the HA-HIF-1α DBD-expressing plasmids at a mass ratio of 10:1 were treated with dexamethasone (100 nM). At the specified time points, ChIP analysis using anti-HA antibody and anti-FLAG antibodies was used to monitor promoter occupancy of the HA-HIF-1α DBD protein and the FLAG-HLG protein, respectively. The immunoenriched DNA samples were detected by agarose gel electrophoresis after PCR (see gels) and quantified by qPCR as well (see graph; the mean of three experiments is shown).
result obtained with full-length protein (Fig. 3B). The half-life of the HA-HIF-1α DBD protein-promoter complexes was no more than 10 min (Fig. 4C). No dissociation of HA-HIF-1α DBD protein was observed upon steroid treatment in cells lacking FLAG-HLG (data not shown), demonstrating that the Fig. 4C result indeed represents capture of the exposed promoter by FLAG-HLG after dissociation of the HIF-1α DBD. ChIP analysis using anti-FLAG antibody showed that the FLAG-HLG fusion proteins rapidly gained access to the promoter, reaching half-saturation at about 10 min as well (Fig. 4C). When this experiment was repeated using the antagonist RU486 in place of dexamethasone, the same result was obtained (supplemental Fig. S8), showing that the result is independent of whether or not the protein has a functional activation domain. We conclude that HRE complexes containing only the HIF-1α DBD are far less stable kinetically than those containing the full-length protein and that they undergo rapid and reversible association and dissociation with the promoter.

To determine if an activation domain is critical for the formation of kinetically stable complexes, as was the case for Gal4 (29), an HA-tagged HIF-1α DBD-VP16 AD fusion protein was created. As expected, cells transfected with a vector that expresses this protein strongly express an HIF-1-responsive reporter gene (Fig. 5A) even when native HIF-1α activity is not induced. This plasmid was then co-transfected into cells along with the FLAG-HLG construct in a mass ratio of 1:10 to again set the stage for a competitive ChIP experiment. As shown in Fig. 5B, this experiment revealed that the HRE-HA-HIF-1α DBD-VP16 complex displayed a half-life of at least 30–60 min, and little or no FLAG-HLG association with the promoter was observed, in stark contrast to the rapid loss of the DBD alone from the promoter in the face of excess competitor FLAG-HLG (Fig. 4C). These data show that a functional activation domain is essential for HIF-1α to form long lived complexes with its cognate promoters.

**HIF-1α Is Cleared from Promoters by Proteasome-mediated Turnover upon Cessation of Signaling**—Having established that HIF-1–promoter complexes are quite stable under inducing conditions, we then turned to the question of how HIF-1 activity is turned off upon cessation of the inducing signal, in this case CoCl2.

HeLa cells were switched back to normal media after having been treated with CoCl2 for 5 h, and the time course of the HIF-1α protein clearance from the VEGF promoter was monitored using a standard ChIP assay. In other words, the cells did not contain FLAG-HLG, so net dissociation of the protein from the promoter was measured rather than reversible dissociation as is the case in the competition ChIP assay. As shown in Fig. 6A, the HIF-1α-dependent ChIP signal decreased gradually with a t1/2 of 30–60 min. As shown in Fig. 6B, this time course was similar to that observed for the decrease in total HIF-1α protein levels as determined by Western blotting.

Since the ubiquitin proteasome pathway is responsible for limiting HIF-1α levels under noninducing conditions, a likely scenario is that after removal of CoCl2, hydroxylation of HIF-1α is reactivated, and the protein level is reduced by VHL-mediated polyubiquitylation and subsequent proteasome-mediated proteolysis. To test this, we examined the effect of the proteasome inhibitor MG132 on clearance of bulk and promoter-bound HIF-1α. HeLa cells were treated with CoCl2 for 5 h and then switched back to media lacking CoCl2 and at this point were also treated with MG132. Western blotting with an anti-HIF-1α antibody showed that the protein levels did not decrease substantially in the MG132-treated cells over the next 3 h. There was an increase in the amount of higher molecular mass species that presumably represent ubiquitylated forms of the HIF-1α protein (Fig. 6C). ChIP analysis showed that MG132 inhibited removal of HIF-1α from the promoter (Fig. 6D). As
mentioned above, in the absence of MG132, promoter-bound HIF-1α is almost undetectable 3 h after the removal of CoCl₂ (Fig. 6A), but in the presence of the proteasome inhibitor, the half-life of the complex was at least 3 h after the removal of CoCl₂ (Fig. 6D). These data show that ubiquitin proteasome pathway-mediated turnover is important for removal of HIF-1α protein from promoters once signaling ceases.

The close correlation between the observed lifetimes of the total and DNA-bound HIF-1α protein populations (Fig. 6) suggests that the proteasome operates on both the free and promoter-bound HIF-1α directly. Alternatively, the data could be explained by a model in which the proteasome attacks only free HIF-1α, but the free and promoter-bound populations of HIF-1α are in rapid exchange. To address the possibility that

HIF-1-promoter binding becomes rapidly reversible after the removal of CoCl₂, we grew cells containing FLAG-HLG in the presence of 150 μM CoCl₂, and then switched them to media lacking the metal complex. Fifteen minutes later, dexamethasone (100 nM) was added to initiate the competition ChIP assay. HeLa cells were treated as described in C, and the level of promoter-bound HIF-1α over time was determined by a standard ChIP assay. The immunoenriched DNA samples were detected by agarose gel electrophoresis after PCR (see gels) and quantified by qPCR as well (see graph; the mean of three experiments is shown).
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![ChIP](image)

**FIGURE 8. Association of the 20 and 19 S proteasome subcomplexes with the VEGF promoter after removal of CoCl₂**. HeLa cells were incubated in medium containing 150 μM CoCl₂ for 5 h and then switched to media lacking CoCl₂. At the indicated time points, ChIP analysis using antibodies against the β1 subunit of the 20 S particle and the Rpt4 subunit of the 19 S particle were used to monitor promoter occupancy of the 20 and 19 S particle, respectively. The immunoenriched DNA samples were detected by agarose gel electrophoresis after PCR (see gels) and quantified by qPCR as well (see graph; the mean of three experiments is shown).

To probe this directly, we measured the association of the proteasome with the VEGF promoter after the removal of the CoCl₂ by ChIP analysis. As shown in Fig. 8 (top gel), the 20 S core particle of the proteasome, as monitored with an anti-β1 subunit antibody, is readily detectable on the VEGF promoter 5 h after treatment of the cells with CoCl₂ and immediately before its removal. This is the “0” time point shown in Fig. 8 (note that the “no antibody” control experiment provided no detectable band (not shown)). The 20 S-dependent ChIP signal increased 2–3-fold over the 2 h following the withdrawal of CoCl₂, consistent with the notion that more proteasome is recruited to the promoter to degrade the HIF-1α protein and/or other transcription factors on the promoter. Interestingly, when the ChIP analysis was repeated using an antibody against Rpt4, a component of the 19 S regulatory particle, the signal was strong at the time of CoCl₂ withdrawal and remained constant. This differential association of the different subunits of the proteasome has been seen previously and probably reflects the proteolysis-independent functions of some of the 19 S regulatory particle proteins in transcription (36–38), as will be discussed below.

*MG132 Blocks HIF-1-mediated Transcriptional Activation*—In concert with these experiments that addressed the role of proteasome-mediated turnover in shutting off HIF-1-mediated transcription after withdrawal of CoCl₂, we also examined the effect of proteasome inhibitor on the system under noninducing conditions. When HeLa cells were treated with 25 μM MG132 in the absence of CoCl₂ (i.e. “normoxic,” noninducing conditions) Western blotting revealed a large increase in the level of HIF-1α protein (supplemental Fig. S9). Indeed, the levels of HIF-1α in the cells treated with MG132 were similar to that observed in CoCl₂-treated cells. This was not surprising, since CoCl₂ and MG132 both block HIF-1α turnover, albeit at different stages. However, in the MG132-treated cells, higher molecular mass forms of HIF-1α were detected that were absent in the CoCl₂-treated cells (Fig. S9), possibly representing polyubiquitinated forms of HIF-1α. The increase in HIF-1α protein also resulted in a corresponding build-up of HRE-HIF-1 complex on the VEGF promoter, as determined by a standard ChIP assay (Fig. S9). However, although the activator was resident on the promoter, MG132 treatment almost completely blunted the activation of a HIF-1α-responsive reporter gene in the presence of CoCl₂ (Fig. S9). This observation suggests that proteasome activity is essential for potent HIF-1α-mediated gene activation at some step downstream of promoter occupancy, consistent with a previous report (39).

**DISCUSSION**

At least two cases have been reported of mutations in transactivators that have little or no effect on the equilibrium binding affinity for a target DNA sequence but increase both the association and dissociation rates in parallel. These mutants are weaker transactivators than the corresponding wild-type proteins (1, 2), arguing that, as one would expect, the duration of transactivator binding to a promoter is an important determinant of gene expression.

Unfortunately, it is difficult to study this important aspect of transactivator function in living cells. A few detailed studies of green fluorescent protein-fused nuclear hormone receptors binding to hundreds of clustered sites in artificial promoters using fluorescence recovery after photobleaching (FRAP) have demonstrated that these proteins form highly dynamic complexes in living cells (11, 40, 41). Unfortunately, FRAP experiments and related techniques require expensive and specialized equipment and are difficult to apply to the study of activator binding to native promoters, except in special cases (26).

To address this methodological need, we developed the competition ChIP assay (29), initially for the study of yeast activators and, in this study, have adapted the assay for use in mammalian cells. As shown in Fig. 1, the technique sets up a competition between an already established, native transactivator-promoter complex and a competitor protein that is introduced into the nucleus at a particular time. The idea is that the competitor protein can only access the promoter if the native factor dissociates. If there is a large excess of the competitor protein relative to the native activator, then this dissociation
event becomes essentially irreversible. Using antibodies that can distinguish the native activator and the competitor protein, the loss of the ChIP signal due to the native activator and the gain of a ChIP signal due to the competitor protein together provide a measure of the dissociation rate of the native activator-promoter complex. The key feature of this system is the use of competitor proteins containing the nuclear hormone ligand-binding domain fused to the DNA-binding domain of the transactivator of interest. In the absence of steroid, these proteins are held in an inactive form by association with Hsp90 (42–45). The addition of steroid to the cells triggers their release, allowing them to compete with the native activator. The competition ChIP assay provides a useful complement to the FRAP technique in that it can be applied easily to the study of native promoters and activators. On the other hand, FRAP can track events that occur on a time scale of seconds (11), whereas the competition ChIP assay used here cannot be used to measure quantitatively events that occur with a half-life of less than about 5 min (Fig. 2D). This is presumably due to the time required for the fusion protein-Hsp90 protein to dissociate and the competitor protein to move into the nucleus, although we have not monitored these events specifically.

The HIF-1-VEGF Promoter Complex Is Kinetically Stable—The applicability of this approach to the study of HIF-1-promoter binding in HeLa cells was validated thoroughly. We demonstrated that the competitor protein, called FLAG-HLG, is functional only after the addition of dexamethasone or RU486, potent agonists and antagonists, respectively, of the GR LBD (Fig. 2). Western blotting showed that FLAG-HLG is expressed at a level at least 10-fold higher than that of native HIF-1α even under inducing conditions (Fig. S1). The addition of steroid has no effect on the binding of the native activator as determined by standard ChIP analysis (Fig. S2). We also showed that HIF-1β is present in excess over both the native HIF-1α protein and the FLAG-HLG competitor protein and that competition between native HIF-1α and FLAG-HLG for HIF-1β does not complicate the interpretation of the results (Figs. 2A and S6). This was important in terms of extending the competition ChIP assay from the simplest case of a homodimeric transcription factor, such as Gal4 (29), to a heterodimeric transcription factor like HIF-1.

When this assay was then employed to examine the dynamics of HIF-1 binding to the VEGF promoter in HeLa cells, we found that the complex is stable kinetically, with a half-life in excess of 1 h (Fig. 3B). This argues against a “hit and run” mechanism (11) for HIF-1 in which the activator rapidly and reversibly associates and dissociates from the promoter. It also seems inconsistent with a mandatory coupling of HIF-1 activity with frequent proteasome-mediated turnover of HIF-1α.

Role of the Proteasome in HIF-1-mediated Transcription—Whereas the competition ChIP data argue strongly against a mandatory coupling of HIF-1 activity to HIF-1α turnover, it is interesting that HIF-1-mediated transcription does appear to depend on proteasome activity, as suggested by the sensitivity of HIF-1-mediated transcription to the proteasome inhibitor MG132 (Fig. S8). This finding is consistent with a recent report by Kaluz et al. (39), who demonstrated that HIF-1-driven expression of the carbonic anhydrase gene is proteasome-dependent. Whereas that study did not include a direct analysis of the lifetime of the HIF-1-promoter complex, as was done here, several pieces of indirect evidence were presented that seemed inconsistent with a requirement for proteasome-mediated turnover of promoter-bound HIF-1. These authors suggested that proteasome-catalyzed destruction of a putative HIF-1-repressing protein might explain the proteasome dependence of carbonic anhydrase expression, but much more work remains to be done to test this or other models. It is important to point out, however, that the data presented here and by Kaluz et al. (39) show that one cannot use sensitivity of an activator to proteasome inhibitors alone as evidence for a requirement for activator turnover.

We also found that proteasome activity is important for removing HIF-1α from promoters once hypoxic signaling (in this case simulated by CoCl2 treatment) ceases. As shown in Fig. 6, withdrawal of the metal ion from the medium results in a slow return to the preinduced state. Bulk HIF-1α levels decay with a half-life of 30–60 min under the conditions employed (Fig. 6B), which corresponds closely to the time frame for net dissociation of HIF-1α from the VEGF promoter as measured using a conventional ChIP assay (Fig. 6A). When CoCl2 was removed and the cells were treated with the proteasome inhibitor MG132, HIF-1α was stabilized, and a significant amount of a higher molecular mass form of HIF-1α accumulated (Fig. 6C), which probably correspond to ubiquitylated species. MG132 also reduced the rate of removal of HIF-1α from the promoter greatly (Fig. 6D). The similar response of both the bulk HIF-1α and promoter-bound HIF-1α to proteasome inhibitor after withdrawal of CoCl2 argues that both of these are direct targets of the proteasome. Alternatively, it could be that the proteasome only degrades free HIF-1α and that the promoter-bound and free pools of the protein are in rapid equilibrium. Two pieces of evidence argue against the latter model and favor the former. First, a competition ChIP experiment conducted after the removal of CoCl2 revealed that the HIF-1α protein does not exchange rapidly under these conditions and that loss of the protein and subsequent association of the FLAG-HLG competitor protein occurs with the same kinetics as overall loss of HIF-1α (Fig. 7). Second, standard ChIP analysis using antibodies against the 20 S proteasome core particle show that 20 S levels on the VEGF promoter increase 2–3-fold upon withdrawal of CoCl2 (Fig. 8), consistent with recruitment of the proteasome in connection with turning gene expression off. In the same experiment, the level of one of the 19 S AAA ATPase proteins on the promoter did not change significantly. This might seem surprising from the viewpoint of a monolithic 26 S proteasome complex, but we and others have shown that these ATPases carry out several proteolysis-independent roles in transcription (23, 36–38, 46–51) and, at least in yeast, can be recruited to promoters independent of the 20 S core particle (36). Thus, our observed results could be explained by parallel dissociation of the 20 S-independent proteasomal ATP complex along with concomitant association of the entire 26 S proteasome as gene expression transitions from the “on” to the “off” state. Of course, it is likely that transcription factors other than just HIF-1α are removed proteolytically from the promoter based on precedent from studies of nuclear hormone regulation.
Stable HIF-1-Promoter Binding

Receptors (9, 18, 19), but the extent of coactivator and general transcription factor turnover at HIF-1-responsive promoters must be addressed in future studies. It is also interesting to note that the proteasome has been recently reported to play an important role in down-regulation of basal (unactivated) transcription of HIV Tat-responsive genes (51). It is possible that the increased association of the 20 S core particle with the VEGF promoter during down-regulation of transcription also reflects this function, although the addition of proteasome inhibitor to uninduced cells does not significantly induce transcription of a HIF-1-responsive reporter gene (Fig. S9).

Taking all of the data together, we suggest that clearance of the HIF-1 from the promoter after cessation of hypoxic signaling does not involve destabilization of HIF-1-promoter complexes but instead is likely to involve a resumption of oxygen-dependent hydroxylation and subsequent ubiquitylation and proteasome-mediated turnover of DNA-bound HIF-1α.

An Activation Domain Is Important for the Formation of Kinetically Stable Activator-Promoter Complexes—The kinetic stability of the HIF-1–promoter complex depended critically on the presence of an activation domain. A fragment of HIF-1α, including the entire DNA-binding and HIF-1β association domains, bound to DNA (see first lane in Fig. 4C), but when the kinetic stability of this protein on the VEGF promoter was investigated using the competition ChIP assay, it was found to exchange rapidly (Fig. 4C). The half-life was no more than 10 min (Fig. 4C, bottom left). However, when this HIF-1α fragment was fused to the potent VP16 activation domain of herpes simplex virus (52), the activator-promoter complex was found to be stabilized greatly (Fig. 5B). It is interesting that much the same result was obtained in our studies of the Gal4 transactivator in yeast (29). This transactivator was found to form kinetically stable complexes with GAL promoters under inducing conditions. But under noninducing conditions, where the Gal80 repressor blankets the Gal4 activation domain (53) or in the case of a truncated activator (Gal4–1–841) that lacked the activation domain, the protein bound to GAL promoters in a highly dynamic fashion with a half-life shorter than could be measured by the competition ChIP assay. These data and those obtained in this study of HIF-1 argue that an activation domain is a major determinant of the kinetic stability of transactivator-DNA complexes. Indeed, even for the dynamic nuclear hormone receptor-promoter interactions, the act of driving transcription appears to stabilize the complex somewhat. The liganded, active ER-α cycles on the promoter on a time scale twice as long as the unliganded, inactive ER-α (9). The interaction of antagonist-bound or mutant androgen receptors with their promoters was also found to be dramatically faster than the interaction between the agonist-bound androgen receptors with the promoters (40). Together, these observations suggest that those transcriptional factors tend to reside on the promoters considerably longer during active transcription.

Although the mechanistic basis of this effect is unclear, several models could be considered. One is that the protein–protein interactions between the activation domain and core promoter-bound transcription factors, such as coactivators contribute to the stability of the complex. Indeed, there is some evidence for this from equilibrium studies of transactivator-promoter interactions in living cells (54, 55). Another possibility is that the activation domain is required for a post-translational modification that stabilizes the activator-promoter complex. We found recently that the proteasomal ATPases, including Sug1/Rpt6 and Sug2/Rpt4, potently destabilize Gal4- and Gal4-VP16-promoter complexes in an ATP-dependent fashion (23). As would be anticipated, this activity strongly represses the function of the activator (23, 56). However, we found that monoubiquitylation of the Gal4 DNA-binding domain blocks this inhibitory effect (23). This monoubiquitylation event is critically dependent on the presence of an activation domain (23), thus providing a second potential mechanism for the importance of this domain in regulating the stability of activator-promoter complexes (6). However, it is important to point out that the extension of this mechanism to HIF-1 is speculative at this time. It is not known if specific monoubiquitylation events are important for HIF-1 activity or if this activator is sensitive to the “stripping” activity of the 19 S ATPases.

CONCLUSION

We have successfully adapted the competition ChIP assay from the study of homodimeric yeast Gal4 protein to the more complex case of a heterodimeric activator in mammalian cells. This provides researchers with a relatively general tool for the analysis of activator-promoter lifetimes on native promoters without the need for complex and expensive instrumentation. The application of this technology to the HIF-1α protein revealed that once induced by CoCl2, the protein, along with HIF-1β, forms a kinetically stable complex with the promoter that has a half-life in excess of 1 h. This result, along with previous studies in yeast (29) and Drosophila (26) argue that although some transcription factors are regularly recycled by the proteasome (9, 10, 18) and some bind in a rapidly reversible fashion to promoters (11, 13, 40), there is also a class of activators that bind stably to their target promoters under inducing conditions. Proteasome activity is, however, required for some as yet uncharacterized event in HIF-1–driven gene expression, and the proteasome is also involved in the clearance of HIF-1α protein from promoters during the return to the uninduced state.

Acknowledgments—We thank Dr. Richard Bruick (University of Texas Southwestern Medical Center) and Dr. Geoffrey Greene (University of Chicago) for kindly providing plasmid vectors. We also thank Dr. Devanjan Sikder for reagents and helpful discussions.

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