p300 relieves p53-evoked transcriptional repression of hypoxia-inducible factor-1 (HIF-1)

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HIF-1 (hypoxia-inducible factor-1), a heterodimeric transcription factor comprising HIF-1 α and HIF-1 β subunits, serves as a key regulator of metabolic adaptation to hypoxia. HIF-1 activity largely increases during hypoxia by attenuating pVHL (von Hippel–Lindau protein)-dependent ubiquitination and subsequent 26 S-proteasomal degradation of HIF-1 α . Besides HIF-1, the transcription factor and tumour suppressor p53 accumulates and is activated under conditions of prolonged/severe hypoxia. Recently, the interaction between p53 and HIF-1 α was reported to evoke HIF-1 α degradation. Destruction of HIF-1 α by p53 was corroborated in the present study by using pVHL-deficient RCC4 (renal carcinoma) cells, supporting the notion of a pVHL-independent degradation process. In addition, low p53 expression repressed HIF-1 transactivation without affecting HIF-1 α protein

amount. Establishing that p53-evoked inhibition of HIF-1 reporter activity was relieved upon co-transfection of p300 suggested competition between p53 and HIF-1 for limiting amounts of the shared co-activator p300. This assumption was confirmed by showing competitive binding of *in vitro* transcription/translation-generated p53 and HIF-1 α to the CH1 domain of p300 *in vitro*. We conclude that low p53 expression attenuates HIF-1 transactivation by competing for p300, whereas high p53 expression destroys the HIF-1 α protein and thereby eliminates HIF-1 reporter activity. Thus once p53 becomes activated under conditions of severe hypoxia/anoxia, it contributes to terminating HIF-1 responses.

Key words: hypoxia, hypoxia-inducible factor-1 α (HIF-1 α), p53, p300, protein degradation, transcriptional regulation.

INTRODUCTION

HIF-1 (hypoxia-inducible factor-1), a heterodimeric transcription factor, senses decreased oxygen availability and responds with enhanced transcription of hypoxia-inducible genes involved in angiogenesis, erythropoiesis and energy metabolism, as well as cell survival decisions [1–3]. HIF-1 comprises HIF-1 α [a basic helix–loop–helix protein containing a PAS domain and is defined by its presence in the *Drosophila* Per and Sim proteins and in the mammalian ARNT (aryl hydrocarbon nuclear translocator) protein] and the ARNT, known as HIF-1 β [4]. Generally, the mRNA of both HIF-1 α and HIF-1 β are permanently expressed. However, under normoxia, HIF-1 α protein is kept at a low, often undetectable, level by continuous degradation via the 26 S proteasome, whereas HIF-1 β protein is constitutively present. Mechanistic considerations to explain accumulation of HIF-1 α , which constitutes the regulated and thus hypoxia-inducible component of the active HIF-1 complex, pointed to a crucial role of HIF prolyl hydroxylases and pVHL (von Hippel–Lindau protein) in HIF-1 α degradation. Elegant work suggested that HIF prolyl hydroxylases sense oxygen and target proline residues at positions 402 and/or 564 of HIF-1 α to hydroxylate them [5–7]. Subsequently, pVHL binds to the hydroxylated protein and marks it for proteasomal degradation by ubiquitination [8,9]. While hydroxylation of proline residues at positions 402 and/or 564 affect protein accumulation, hydroxylation of Asn⁸⁰³ regulates transactivation of HIF-1.

Previously, it has been proposed that prolonged exposure to hypoxia activates feed-back mechanisms to decrease HIF-1 α protein amount [10,11]. Mechanistically, this has been attributed to attenuated Akt phosphorylation and subsequent glycogen syn-

thase kinase 3 β [12] or forkhead transcription factor FOXO4 activation [13]. Alternatively, up-regulation of HIF prolyl hydroxylases may limit HIF-1 α protein amount [8,11,14,15]. Other reports, however, presume suppression of HIF-1 α by p53 [16–19], although a detailed description of p53–HIF-1 α interactions in terms of signalling consequences remain elusive.

There is unquestionable evidence that p53 accumulates under conditions of severe/prolonged hypoxia. Findings to explain p53 accumulation point to a decrease in the ubiquitin E3 ligase Mdm2 (mouse double minute 2) [20] or ATR-dependent Ser¹⁵ phosphorylation of p53 [21], the two mechanisms not being mutually exclusive. Accumulation of p53, in turn, may provoke HIF-1 α degradation by formation of a tertiary complex between HIF-1 α , p53 and Mdm2 [17]. Moreover, p53 represses transcriptional activity of HIF-1 [19]. If one assumes competition for limiting cofactors, it is interesting to note that p300 is required for HIF-1, as well as p53 transactivation [22–24]. The requirement of the co-activator p300 for full transcriptional HIF-1 activity was recently appreciated as a potential target for HIF-1 activity regulation [25,26]. Considering that transcriptionally inactive p53, mutated in its DNA-binding domain, retains the ability to block HIF-1 transactivation, whereas a double mutant defective for DNA as well as p300 binding did not inhibit HIF-1, implies that transrepression of HIF-1 by p53 may result from competition for p300 [18].

In order to define mechanisms used by p53 to attenuate HIF-1 transactivation, we used transient overexpression of p53 and elucidated the impact on HIF-1 α protein level and HIF-1 transcriptional activity with some emphasis on the role of p300. Our data suggest that low-level p53 expression attenuates HIF-1 transcriptional activity by competing with limiting amounts of p300, because overexpression of p300 relieves inhibition. Moreover,

Abbreviations used: Epo, erythropoietin; HA, haemagglutinin; HIF-1, hypoxia-inducible factor-1; HRP, horseradish peroxidase; IVTT, *in vitro* transcription/translation; Mdm2, mouse double minute 2; PIM, protease inhibitor mix; PLSD, protected least-squares difference; pVHL, von Hippel–Lindau protein; RCC4, renal carcinoma; RKO, colon carcinoma; URL, unprogrammed reticulocyte lysate.

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competition of HIF-1 α and p53 for p300 was confirmed *in vitro*. Furthermore, high-level p53 expression also decreases the protein amount of HIF-1 α .

EXPERIMENTAL

Materials

All chemicals were of the highest grade of purity commercially available. Specifically, fetal bovine serum was purchased from Biochrom (Berlin, Germany), medium and supplements came from PAA (Linz, Austria). A protein-assay kit was bought from Bio-Rad (München, Germany). PIM (protease inhibitor mix) and a chemiluminescent SeAp reporter gene assay kit came from Roche (Mannheim, Germany). Isopropyl β -D-thiogalactoside was from Biomol (Hamburg, Germany). Nitrocellulose membrane, enhanced chemiluminescence detection system, HRP (horse-radish peroxidase)-labelled anti-mouse or anti-rabbit secondary antibodies and Amplify™ Fluorographic Reagent were from Amersham Pharmacia Biotech (Braunschweig, Germany). Luciferase lysis buffer, luciferase assay reagent and T_NT T7-coupled reticulocyte lysate system were purchased from Promega (Mannheim, Germany). The anti-HIF-1 α antibody was purchased from Becton Dickinson (Heidelberg, Germany), the anti-p53 antibody (FL-393) from Santa Cruz (Heidelberg, Germany), the anti-HA (haemagglutinin) antibody from Covance (Richmond, CA, U.S.A.), the anti-actin antibody and GSH-agarose from Sigma (Steinheim, Germany). pGL-Epo-HRE-luc plasmid, as described previously [27], was kindly provided by Dr T. Kietzmann (Institute of Biochemistry and Molecular Cell Biology, University of Göttingen, Germany). Bax-luciferase plasmid, as described previously [28,29], was kindly provided by Dr K. H. Scheidtmann (Institute for Genetics, University of Bonn, Germany). SeAp reporter plasmid containing a constitutively active promoter [30] was a gift from Dr A. Levitzki (Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Israel). pcDNA3-HIF-1 α (1-826) plasmid was kindly given by Dr P. J. Ratcliffe (Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, U.K.). pcDNA3-HA-p53 plasmid, as described previously [31], was kindly provided by Dr K. H. Vousden (Regulation of Cell Growth Laboratory, National Cancer Institute at Frederick, Frederick, MD, U.S.A.). pDrive mGAPDH (glyceraldehyde-3-phosphate dehydrogenase) was constructed in our laboratory. CMV β -p300-CHA plasmid was kindly provided by Dr A. Hecht (Institute of Molecular Medicine and Cell Research, University of Freiburg, Germany). pGex-4T1 and GST-p300-CH1-pGex-4T1 were a gift from Dr E. Metzen (Institute of Physiology, Medical University of Luebeck, Germany). pcDNA3 control plasmid was purchased from Invitrogen (Karlsruhe, Germany).

Cell culture

Human RKO (colon carcinoma) cells and RCC4 (renal carcinoma) cells were cultured in Dulbecco's modified Eagle's medium with 4.5 g/l D-glucose. Medium was supplemented with 10 % fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin. RCC4 cell medium also contained 1 mM sodium pyruvate. Cells were replated twice a week, and medium was changed prior to experiments. Cells were kept in a humidified atmosphere of 5 % CO₂ in air at 37 °C. Hypoxia (0.5 % O₂/5 % CO₂/94.5 % N₂) incubations were performed in a 3-gas incubator IG750 (Jouan, Unterhaching, Germany), and anoxia (0 % O₂/5 % CO₂/95 % N₂) treatments were carried out in a flow-through manner in plexiglass chambers connected to

a DIGAMIX 5KM 402 gas pump (Woesthoff GmbH, Bochum, Germany).

Cell transfection and reporter assay

For Western blot analysis, 1×10^6 RCC4 cells were seeded in 6 cm or 1×10^6 RKO cells in 10 cm dishes one day prior to transfection. At a rate of approx. 60 % confluency, cells were transfected with 4, 5 or 8 μ g of p53 expression plasmid using PolyFect® transfection reagent (Qiagen, Hilden, Germany), following the manufacturer's instructions. After transfection (16 h), medium was changed and cells were stimulated as indicated. For luciferase reporter assays, 1×10^5 RKO cells were seeded in 6-well plates one day prior to transfection with 0.005, 0.05 or 0.5 μ g of p53-expression plasmid. Cells were co-transfected with 1 μ g of p300-expression plasmid and either Epo (erythropoietin)-luciferase or Bax-luciferase plasmids (0.5 μ g of each) using the same protocol. Following incubation, medium was removed, plates were washed with PBS, and the cells were scraped off in $1 \times$ luciferase lysis buffer. After vortexing for 10 s, cells were incubated for 10 min on ice and centrifuged for 2 min at 12 000 g. The supernatant (10 μ l) was mixed with 100 μ l of luciferase assay reagent and luciferase activity was measured with a LB 9502 luminometer (Berthold, Pforzheim, Germany). For control experiments, cells were transfected with 0.5 μ g of SeAp reporter plasmid. Following incubations, the medium was centrifuged, and subsequently handled according to the manufacturer's instructions.

Western blot analysis

HIF-1 α , p53, HA-tagged p53 and actin were quantified by Western blot analysis. Briefly, cells were incubated, scraped off, lysed in 150 μ l of lysis buffer A (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5 % Nonidet P-40, 1 mM PMSF, $1 \times$ PIM, pH 7.5), sonicated, vortexed (3×10 s), and incubated for 10 min on ice, followed by centrifugation (15 000 g, 30 min). Protein (80 μ g) and 10 μ l of $4 \times$ SDS/PAGE sample buffer (125 mM Tris/HCl, 2 % SDS, 20 % glycerine, 1 mM dithiothreitol, 0.002 % Bromophenol Blue, pH 6.9, in water) were mixed, made up to 40 μ l with water and boiled for 5 min. Proteins were resolved by SDS/PAGE (7.5 % gel). Gels were washed with blotting buffer (25 mM Tris, 192 mM glycine, 20 % methanol, pH 8.3) for 5 min. Proteins were blotted on to nitrocellulose by a semi-dry transfer cell (Bio-Rad), and unspecific binding was blocked with 5 % milk/TTBS (50 mM Tris/HCl, 140 mM NaCl, 0.05 % Tween-20, pH 7.2) for 1 h. Anti-HIF-1 α (1:1000 in 5 % milk/TTBS), anti-p53 (1:1000 in 5 % milk/TTBS), anti-HA (1:1000 in 5 % milk/TTBS) or anti-actin (1:500 in 5 % milk/TTBS) antibody was added and incubated overnight at 4 °C. Afterwards, nitrocellulose membranes were washed 3 times for 5 min each with PBS. For protein detection, blots were incubated with a HRP-labelled goat anti-mouse secondary antibody (1:2000 in 5 % milk/TTBS) or HRP-labelled goat anti-rabbit secondary antibody (1:2000 in 5 % milk/TTBS) for 1 h, washed 3 times for 5 min each with TTBS and once for 5 min with PBS, followed by enhanced chemiluminescence detection.

IVTT (*in vitro* transcription/translation) assay

pcDNA3-HA-p53 and pcDNA3-HIF-1 α (1-826) plasmids were used for IVTT of p53 and HIF-1 α . Proteins were expressed in a T_NT SP6/T7-coupled reticulocyte lysate system in the presence of [³⁵S]methionine (ICN Biomedicals, Eschwege, Germany). Proteins were resolved by SDS/PAGE (10 % gel). Gels were

fixed in fixing solution (50 % methanol, 10 % acetic acid, 40 % water), incubated in Amplify™ Fluorographic Reagent, dried and exposed to X-ray films for 8–16 h.

Preparation of GST (glutathione S-transferase)-fusion proteins

pGEX-4T1 (control) and pGEX-4T1 p300-CH1 (p300-CH1) plasmids were transformed into *Escherichia coli* DH5.1α cells. Bacteria were grown in 200 ml of Luria–Bertani medium with 50 µg/ml ampicillin to reach a D_{600} of 0.6–0.8, and protein expression was induced with 0.5 mM isopropyl β-D-thiogalactoside for 3 h. After centrifugation, cells were resuspended in 10 ml of lysis buffer B (1 % Triton-X100, 1.5 % *N*-laurylsarcosine, 25 mM triethanolamine, 1 mM EDTA in PBS), supplemented with lysozyme (1 mg/ml), and incubated for 10 min on ice. 1 × PIM and 1 mM PMSF were added, followed by sonification. After centrifugation (10 000 *g*, 4 °C, 20 min), the protein-containing supernatant was incubated with 300 µl of 50 % slurry of GSH-agarose overnight at 4 °C. After washing 5 times with excess 1 % (v/v) Triton X-100 in PBS, the protein bound to GSH-agarose was resuspended in 500 µl of 1 % Triton X-100 in PBS. Proteins bound to GSH-agarose were verified using SDS/PAGE (10 % gels), followed by Coomassie Blue staining.

Competition binding assay

Protein-bound GSH-agarose (10 µl; either control or p300-CH1), 40 µl of ³⁵S-labelled HIF-1α and/or ³⁵S-labelled p53 (1 to 100 µl) were mixed in NET-N buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.5 % Nonidet P-40, 1 × PIM, 1 mM PMSF, pH 8.0). URL (unprogrammed reticulocyte lysate), i.e. the IVTT mix without addition of a protein-encoding plasmid, was used as a control (10 or 100 µl). To increase the visibility of the pellet, 40 µl of equilibrated GSH-agarose was added. Incubations were performed for 16 h at 4 °C. Following centrifugation (500 *g*, 5 min), 20 µl of the supernatant were mixed with 20 µl of 4 × SDS/PAGE sample buffer and boiled at 95 °C for 5 min. The agarose pellet was washed 5 times with NET-N buffer, subsequently mixed with 40 µl of 4 × SDS/PAGE sample buffer and boiled at 95 °C for 5 min. Protein separation and detection was similar to the procedure described for IVTT.

Statistical analysis

Each experiment was performed at least three times and representative experiments are shown. The results are expressed as the means ± S.D. A Fisher's PLSD (protected least-squares difference) test of an ANOVA was used to determine statistically significant differences.

RESULTS

Impact of p53 on HIF-1α protein accumulation

It is widely accepted that HIF-1α accumulates under conditions of decreased oxygen tension, with the notion that HIF-1α decreases after prolonged periods of hypoxia. This is attributed, among other possibilities, to p53 and HIF-1α interactions that promote HIF-1α degradation [17].

To assess the impact of p53 on down-regulation of HIF-1α under experimental conditions of prolonged hypoxia, we used RKO cells, which contain wild-type p53. Cells were time-dependently exposed for 2 to 24 h of anoxia. Anoxia caused strong HIF-1α accumulation with maximal levels around 8 h, followed by a slight protein decrease at 24 h (Figure 1). p53 became detectable at 16 h with a stronger response seen at 24 h.

Figure 1 Expression of HIF-1α under anoxia in combination with p53 overexpression

HIF-1α protein accumulation was determined in parent RKO cells (w/o transfection) or p53-overexpressing RKO cells, following exposure to increasing periods of anoxia. HIF-1α, p53, HA-p53 and actin protein levels were determined by Western blotting analysis. Blots are representative for at least 3 independent experiments.

Figure 2 HIF-1α stability in combination with p53 overexpression in RCC4 cells

HIF-1α, HA-p53, endogenous plus exogenous p53 (p53) and actin levels were determined by Western blotting analysis, following 24 h of normoxia or anoxia in RCC4 cells. Cells were untransfected or transfected with 4 µg or 8 µg of p53-expression plasmid. Blots are representative for at least 3 independent experiments.

In another set of experiments, cells were transfected with a p53-expression plasmid, resulting in transient p53 overexpression/stabilization, verified by detecting the HA-tag of exogenous p53. In addition, tagged p53 runs slower than endogenous p53 due to an increased molecular mass of the tag. Exposing these cells for 2 to 24 h of anoxia resulted in HIF-1α protein expression. However, HIF-1α expression was transient with maximal expression at 8 h. Thereafter, protein disappeared much faster at 16 and 24 h compared with experiments in parent RKO cells. At 16 and 24 h, expression of endogenous p53 was equivalent to the situation seen in parent RKO cells.

To verify that reduction of HIF-1α protein indeed resulted from p53 expression, we repeated experiments in RCC4 cells, which constitutively express HIF-1α due to a defect in pVHL and thus impaired 26 S-proteasomal degradation of HIF-1α. Overexpression of p53 lowered HIF-1α protein expression upon transfection with 4 µg or 8 µg of p53-expressing plasmids (Figure 2).

HIF-1α disappearance was more pronounced under anoxia compared with normoxia, which correlates with endogenous p53 accumulation under anoxia, but not normoxia. It can be concluded that expression of p53 attenuates HIF-1α accumulation or down-regulates the amount of HIF-1α, once the protein is stabilized due to the absence of pVHL. Apoptotic events were excluded under condition of p53 expression, because neither an increase in caspase-3-like activity nor a loss of mitochondrial

Figure 3 HIF-1 transcriptional activity during hypoxia and p53 expression

(A) RKO cells transfected with 0.5 µg of Epo-luciferase plasmid were exposed for 24 h to either normoxia or hypoxia under conditions of co-transfection with 0.5 µg of control plasmid (pcDNA3, open columns) or 0.5 µg of p53-expression plasmid (p53, closed columns). Relative Epo-luciferase activity is normalized to control plasmid transfected cells incubated for 24 h under normoxia. Results are expressed as the means $\pm$ S.D., $n \geq 4$ (ANOVA: $F = 64.205$, $P < 0.0001$; Fisher's PLSD test: $***P \leq 0.0001$). (B) RKO cells transfected with 0.5 µg of SeAp reporter plasmid were exposed for 24 h to hypoxia under conditions of co-transfection with 0.5 µg of control plasmid (pcDNA3, open columns) or 0.5 µg of p53 expression plasmid (p53, closed columns). Relative SeAp activity is normalized to cells transfected with corresponding amounts of control plasmid.

membrane potential, analysed using 3'-dihexyloxycarbocyanine iodide staining and FACS analysis, was detected in normoxic compared with anoxic cells (results not shown).

Impact of p53 on HIF-1 transcriptional activity

Considering that the impact of p53 on HIF-1 may not only be seen at the HIF-1 α protein level, we went on to investigate HIF-1 transactivation. Therefore, RKO cells were co-transfected with p53-expression plasmids, pcDNA3 control plasmid and the pGL-Epo-HRE plasmid (Epo-luciferase), which has three copies of the Epo hypoxia-responsive element (HRE) in front of a luciferase construct, as described previously [27]. Thus luciferase activity is a measure for HIF-1 transactivation. Since hypoxia (0.5% O₂, 24 h), in contrast with anoxia (0% O₂, 24 h), did not induce endogenous p53 expression (results not shown) [32], experiments were performed under hypoxic conditions to ensure that modulation of HIF-1 transactivation exclusively resulted from exogenous p53. Hypoxia caused a 2.5 ± 0.6 fold induction of Epo-luciferase activity compared with normoxia (Figure 3A).

Transient overexpression of p53 attenuated HIF-1 transcriptional activity by approx. 75% under normoxic conditions, i.e. p53 blocked basal reporter activity. Importantly, under hypoxia,

expression of p53 substantially lowered Epo-luciferase activity. p53 reduced HIF-1 transactivation below control, i.e. normoxic values. Differences reached statistical significance (ANOVA: $F = 64.205$, $P < 0.0001$; Fisher's PLSD: $P \leq 0.0001$). To exclude unspecific effects of p53, a constitutively active SeAp reporter plasmid was co-transfected with either a p53-expression plasmid or the pcDNA3 control plasmid. p53 did not inhibit SeAp activity compared with the control plasmid (Figure 3B). Apparently, p53, besides affecting HIF-1 α stability under anoxia, also reduced HIF-1 transcriptional activity under hypoxic conditions. An interference of p53 with the transcriptional activity of HIF-1 may result from competition for limiting co-activators, such as p300. Initial experiments in a hepatocyte cell line (HepG2) indicated suppression of endogenous Epo mRNA expression by p53.

Impact of p300 on p53-mediated HIF-1 transcriptional repression

The transcriptional co-factor p300 is required for full activity of both HIF-1 and p53. To test whether limiting p300 explains impaired HIF-1 transactivation under the impact of p53, RKO cells were co-transfected with increasing amounts of p53 or pcDNA3 control plasmid and a p300 expression plasmid (1 µg), in addition to the Epo-luciferase reporter plasmid (0.5 µg). Hypoxia (0.5% O₂, 24 h)-evoked HIF-1 transcriptional activity decreased with co-transfection of increasing amounts of p53 expression plasmid (Figure 4A).

The p53-expression plasmid, 0.005 µg, reduced HIF-1 activity significantly to 0.67 ± 0.06 -, 0.05 µg to 0.32 ± 0.09 - and 0.5 µg to 0.27 ± 0.10 -fold compared with the activity of respective control plasmid transfected cells (P values of 0.0003, < 0.0001 and < 0.0001 respectively). Furthermore, transfection of 0.05 µg and 0.5 µg of p53 plasmid significantly decreased HIF-1 activity compared with cells transfected with 0.005 µg of p53 plasmid ($P = 0.002$; $P \leq 0.0001$).

Co-transfection of p300 altered Epo-luciferase activity significantly, partially reversing inhibition seen with p53. Co-transfection of 0.005 µg of p53 and p300 provoked 1.52 ± 0.21 -fold stimulation of luciferase activity compared with the inhibition seen with p53 alone, the difference being significant ($P < 0.0001$). Co-transfection of p300 in the presence of 0.05 µg of p53 plasmid significantly reversed the inhibition seen with p53 alone ($P = 0.0114$). Importantly, inhibition by p53 was approx. 70%, whereas co-transfection of p300 reduced this value to approx. 50%. Increasing the amount of p53-expression plasmid to 0.5 µg eliminated the action of p300 in reversing inhibition. We conclude that p53-mediated HIF-1 inhibition can be antagonized by p300, though at low level of p53 expression only. To ensure that p300 is indeed a limiting factor for both HIF-1 and p53 transcriptional activity, RKO cells were either co-transfected with an Epo-luciferase plasmid (0.5 µg) and 1 µg of p300-expression plasmid and incubated under hypoxic conditions for 24 h, or co-transfected with plasmids expressing Bax-luciferase (0.5 µg), p53 (0.5 µg) and p300 (1 µg). Both HIF-1 and p53 reporter activity significantly increased upon co-expression of p300 (Figure 4B). Since the described p300-mediated relief of the p53-evoked HIF-1 transactivity inhibition could also be a stimulatory effect of p300 on HIF-1 transactivity without necessarily implying a competition between p53 and HIF-1 for p300, we used an *in vitro* competition binding assay to ensure that indeed a direct competition between p53 and HIF-1 α for binding to p300 is the underlying mechanism.

In order to confirm that exogenous and anoxia-evoked endogenous p53 are active, RKO cells were transfected with a Bax-luciferase reporter plasmid containing p53 response elements [28], followed by exposure to hypoxia, anoxia or co-transfection

Figure 4 HIF-1 transcriptional activity in the presence of p300 and p53

(A) RKO cells were exposed to hypoxia for 24 h under conditions of transfection with indicated amounts of control plasmid (pcDNA3, open columns), p53-expression plasmid (p53, closed columns) and p53/p300 (1 µg) co-expression (p53 + p300, hatched columns). Relative Epo-luciferase activity is normalized to control plasmid transfected cells exposed to hypoxia for 24 h. (B) RKO cells were exposed for 24 h to hypoxia with or without co-transfection of 1 µg of p300-expression plasmid. Relative Epo-luciferase activity is normalized to cells without p300 transfection. Alternatively, cells were transfected with plasmids allowing expression of p53 (0.5 µg), Bax-luciferase and/or p300 (1 µg). Relative Bax-luciferase activity is normalized to cells transfected with p53 only. The results are expressed as the means $\pm$ S.D., $n \geq 4$ (ANOVA: $F = 48.726$, $P < 0.0001$).

with plasmids forcing p53 expression. Overexpression of p53 caused a 4.2 ± 0.5 -fold increase in Bax-luciferase activity compared with cells transfected with Bax-reporter alone. Anoxia (0% O₂, 24 h) evoked p53 stabilization and induced Bax-luciferase activity 3.0 ± 0.6 -fold compared with normoxia-treated cells, whereas hypoxia did not induce any Bax-luciferase activity.

In vitro competition between p53 and HIF-1 α for binding to p300

To demonstrate direct competition between p53 and HIF-1 α for binding to p300, we employed an *in vitro* competition assay. Therefore, IVTT in the presence of [³⁵S]methionine was used to generate ³⁵S-labelled p53 and ³⁵S-labelled HIF-1 α proteins. Subsequently, these proteins were incubated with bacterially expressed p300 CH1 domain bound to GSH-agarose. Only the CH1 domain of p300 was used, since this region had been identified previously to contain a binding site for both p53 and HIF-1 α [24,33]. Increasing concentrations of p53 reduced binding of HIF-1 α to p300 CH1 domain, whereas URL displayed no such effect on HIF-1 α binding (Figures 5A and 5B).

Relative densitometric analysis of HIF-1 α bound to p300 gave a decrease from starting values of 100% to values around 30–40%. To exclude that p53 interacts directly with HIF-1 α , ³⁵S-labelled p53 was incubated with a peptide-spot-array membrane

Figure 5 Competitive binding of HIF-1 α and p53 to p300

(A) IVTT-generated ³⁵S-labelled p53 and/or ³⁵S-labelled HIF-1 α , and (B) ³⁵S-labelled HIF-1 α and/or URL were incubated at different ratios with p300-CH1 bound GSH-agarose. Subsequently, the pull-down and the supernatant, as loading control, were analysed for bound p53 or HIF-1 α by autoradiography. HIF-1 α pulled down by p300-CH1 bound GSH-agarose was determined by densitometry.

containing 272 overlapping peptide fragments of HIF-1 α , each 15 amino acids in length, with an offset of 3 amino acid residues. Since no binding of p53 to HIF-1 α was detected (results not shown), we concluded that reduced binding of HIF-1 α to p300 with increasing amounts of p53 can entirely be attributed to competition with p300.

DISCUSSION

We showed down-regulation of anoxia-evoked HIF-1 α accumulation by p53. HIF-1 α degradation was shown to be pVHL-independent by confirmatory results obtained in RKO cells and also in pVHL-deficient RCC4 cells. Hypoxic transactivation of HIF-1 was attenuated by p53, an effect relieved by overexpression of p300. Based on *in vitro* binding assays, we predict competition between HIF-1 α and p53 for binding to limiting amounts of the shared co-activator p300.

HIF-1 α accumulation and transactivation of HIF-1 constitutes the master regulatory system to cope with conditions of reduced oxygen availability [34], although it is known to be activated by other factors, such as growth factors, hormones or nitric oxide [1,2,35–37]. The term hypoxia, or low oxygen tension, as the physiological stimulus for HIF-1, is sometimes used imprecisely to describe conditions of reduced oxygen availability as low as anoxia, either in the presence or absence of nutrients, e.g. serum. HIF-1 α accumulation and HIF-1 transactivation seem to be similarly induced under most of these conditions, but other responses towards hypoxia, such as p53 accumulation, show a marked dependency on the degree of hypoxia and/or its duration.

In some systems, 6 h of anoxia suffice to accumulate p53 [38,39], whereas other studies required 48 h [40]. Transactivation of p53 also revealed discrepancies. Some studies suggest transactivation of p53 by anoxia [41], whereas others showed a stabilized, but transcriptionally inactive, p53 protein [42]. As seen in our present study, prolonged periods of anoxia beyond 16 h caused accumulation of transcriptionally active p53 in RKO cells, as determined by a Bax-luciferase reporter system. In contrast, hypoxia (0.5 % O₂) neither stabilized p53 nor its transcriptional activation.

Previously, p53 has been shown to down-regulate HIF-1 α protein by reducing its half-life [19]. Based on a peptide-spot membrane, a direct interaction between the core domain of p53 and HIF-1 α was shown [43], but more recent evidence suggests that Mdm2 is required to bridge the two proteins, thereby facilitating HIF-1 α degradation [17]. Destruction of HIF-1 α by p53 is corroborated by our findings showing that transient overexpression of p53 markedly reduced HIF-1 α protein. Anoxia accelerated this process, most likely due to activation of endogenous p53, as seen in RCC4 cells (Figure 2). It is of interest that overexpression of p53 reduced, but did not abolish the initial phase of HIF-1 α accumulation in RKO cells under anoxia (Figure 1), while stabilization of endogenous p53 further induced degradation of HIF-1 α . Experiments in RCC4 cells provided unequivocal proof that p53 uses a pVHL-independent mechanism to destabilize HIF-1 α , which may support the concept of the E3 ligase Mdm2 being capable of ubiquitinating and degrading HIF-1 α [19]. The HIF-1 α –Mdm2–p53 system can be considered a feed-back loop to limit HIF-1 responses under prolonged hypoxia/anoxia [10–12].

Besides affecting HIF-1 α protein and thus, indirectly hindering HIF-1 α –HIF-1 β -complex formation, as well as target gene activation, direct inhibition of HIF-1 transcriptional activity by p53 constitutes an alternative level of regulation. Attenuated HIF-1 transcriptional activity by p53 has been suggested previously [16,19]. We confirmed these observations in RKO cells and suggest limiting amounts of the transcriptional co-activator p300 as a mechanistic explanation [44–47]. The binding site for both p53 and HIF-1 α at p300 is the CH1 domain, although p53 additionally complexes with the CH3 domain [24]. Our experiments revealed that reporter activity of p53, as well as HIF-1, are enhanced by co-transfection of p300, supporting the notion that p300 is limiting for both transcription factors. Inhibition of HIF-1 reporter, facilitated by low-level p53 expression, was relieved by co-transfection of p300. Since experiments *in vitro* showed competition of p53 and HIF-1 α for binding to p300, we presume that the same mechanism operates in cells. However, p300 did not antagonize inhibition of HIF-1 reporter activity when used in combination with p53 expression rates shown to affect HIF-1 α protein level. This implies that, depending on relative p53 expression, distinct mechanisms suppress HIF-1 (Figure 6). Hypoxic conditions cause HIF-1 α accumulation and HIF-1 transactivation. Under more severe conditions, i.e. anoxia, p53 accumulates and competes with HIF-1 α for binding to p300, which results in HIF-1 transcriptional repression. With enduring treatment, amounts of p53 increase, which in turn facilitates HIF-1 α degradation.

This model is supported by findings that p53 mutated in its p300-binding domain failed to block HIF-1 transactivation [18]. In contrast to our present study the same group could not relieve p53-induced HIF-1 transactivation inhibition by p300 overexpression [16]. These differences can be explained by the amount of p53 being transfected, as p300 releases inhibition only in combination with low p53 expression.

We conclude that transcriptional activity of HIF-1 not only depends on protein stability of HIF-1 α , but also on cofactor, i.e.

Figure 6 Proposed HIF-1–p53 interactions

Hypoxic induction of HIF-1 transactivation is repressed under conditions of prolonged anoxia due to p53 accumulation and competition for p300. Under enduring anoxia, p53 protein levels further increase and promote pVHL-independent HIF-1 α destruction. For details, see the text.

p300, availability. Our results provide further evidence for the importance of p53 in modulating HIF-1 responses.

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