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## Cyclosporin A Prevents the Hypoxic Adaptation by Activating Hypoxia-inducible Factor-1 $\alpha$ Pro-564 Hydroxylation\*

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The mechanism by which hypoxia induces gene transcription involves the inhibition of hypoxia-inducible factor (HIF)-1 $\alpha$  prolyl hydroxylase activity, which prevents von Hippel-Lindau (vHL)-dependent targeting of HIF-1 $\alpha$  to the ubiquitin-proteasome pathway. HIF-1 $\alpha$  is stabilized, translocates to the nucleus, interacts with hypoxia-responsive elements, and promotes the activation of target genes. This report shows that cyclosporin A (CsA) interferes with the hypoxic signaling cascade in C6 glioma cells. CsA inhibits hypoxia-dependent gene transcription in a reporter gene assay and prevents the hypoxic accumulation of HIF-1 $\alpha$ . Addition of the 530–603 C-terminal oxygen-dependent degradation (ODD) domain of HIF-1 $\alpha$  to the green fluorescent protein (GFP) destabilized the protein in an oxygen-dependent manner. CsA prevented the hypoxic stabilization of an ODD-GFP fusion protein. An assay for 2-oxoglutarate-dependent dioxygenases was developed using a light mitochondrial kidney fraction as a source of enzyme. It uses the capacity of specific peptides to stimulate the degradation of [ $^{14}\text{C}$ ]2-oxoglutarate. CsA stimulated the enzymatic activity in the presence of a peptide that mimicked the 557–576 sequence of HIF-1 $\alpha$ . The enzyme promoted [ $^{35}\text{S}$ ]vHL binding to glutathione S-transferase (GST)-ODD fusion protein. This association increased in the presence of CsA. CsA effects were not observed when the proline residue corresponding to Pro-564 in the HIF-1 $\alpha$  sequence was replaced by a hydroxyproline or an alanine residue. Finally, CsA increased vHL-ODD interaction during hypoxia. We conclude that CsA destabilizes HIF-1 $\alpha$  by promoting hydroxylation of Pro-564 in the ODD domain. Such a mechanism may prevent hypoxic adaptation during CsA-induced nephrotoxicity and contribute to the adverse effects of this drug.

Cells respond to low oxygen tensions by up-regulating the expression of genes involved in angiogenesis (e.g. vascular endothelial growth factor), erythropoiesis (e.g. erythropoietin), and glycolysis. The transcriptional activation of target genes is induced by a common transcription factor, hypoxia-inducible factor-1 (HIF-1).<sup>1</sup> HIF-1 was first identified as a heterodimeric

transactivator that recognizes a specific DNA sequence, termed hypoxia-responsive element (HRE) in the 3'-untranslated region of the erythropoietin gene (1). HIF-1 consists of two subunits, HIF-1 $\alpha$  and the aryl hydrocarbon receptor nuclear translocator, both of which belong to the large family of basic helix-loop-helix-*per-arnt-sim* transcription factors (2, 3). Under normoxic conditions, HIF-1 $\alpha$  subunits are unstable, being rapidly targeted to the ubiquitin-proteasome pathway. Degradation is mediated by a ubiquitin-protein isopeptide ligase (E3) complex, in which the von Hippel-Lindau protein (vHL) binds to a specific hydroxylated proline residue (Pro-564) within the oxygen-dependent degradation (ODD) domain of HIF-1 $\alpha$  (4, 5). A major action of hypoxia is to suppress prolyl hydroxylation and degradation of HIF-1 $\alpha$  by the proteasome. As a consequence, the protein accumulates, migrates to the nucleus, and associates with the aryl hydrocarbon receptor nuclear translocator, and the complex interacts with the HRE of target genes (6, 7). The importance of this mechanism is ascertained by the facts that vessel formation is hampered in HIF-1 $\alpha$   $-/-$  knock out mice (8) and that mutations in the *vHL* gene cause hereditary cancer syndrome associated with dysregulated angiogenesis and up-regulation of hypoxia-sensitive genes.

Cyclosporin A (CsA) is a potent immunosuppressive agent used after organ transplantation and in the treatment of several autoimmune diseases. CsA is well known to be nephrotoxic probably as a consequence of the constriction of renal vessels and of the resulting hypoxia. Circumstantial evidence further suggests that CsA interferes with the hypoxic signaling cascade. (i) Maruyama *et al.* (9) reported that CsA inhibited vascular endothelial growth factor production by human lymphocytes. (ii) CsA inhibits erythropoietin production in anemic mice (10). (iii) Kang *et al.* (11) reported that vascular endothelial growth factor reverses some of the post-CsA-mediated hypertension and nephropathy in the rat. (iv) Erythropoietin deficiency has been proposed as a cause of the anemia observed in children following cardiac transplantation (12). These would suggest that CsA inhibits the cellular responses that are mediated by HIF-1 $\alpha$  and HRE.

In the present study, we examine the possible influence of CsA on the hypoxic signaling. Results show that CsA inhibits hypoxia-induced gene transcription by preventing hypoxia-induced and ODD-dependent stabilization of HIF-1 $\alpha$ . We also show that CsA stimulates a kidney prolyl hydroxylase activity that specifically modifies Pro-564 in the ODD domain of HIF-1 $\alpha$ .

### MATERIALS AND METHODS

**Materials**—Culture medium, restriction, and DNA-modifying enzymes were from Promega. Culture media and fetal calf serum were

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<sup>1</sup> The abbreviations used are: HIF, hypoxia-inducible factor; 2-OG, 2-oxoglutarate; CsA, cyclosporin A; GFP, green fluorescent protein;

GST, glutathione S-transferase; HRE, hypoxia-responsive elements; ODD, oxygen-dependent degradation; PHD, prolyl hydroxylase; vHL, von Hippel-Lindau; Luc, luciferase; PBS, phosphate-buffered saline.

from Invitrogen. All chemicals were purchased from Sigma. Mouse monoclonal antibodies were obtained from the following sources: HIF-1 $\alpha$  (Novus Biologicals), GFP (Roche Molecular Biochemicals),  $\alpha$ -tubulin (Sigma), vHL (Pharmingen). HIF peptides were synthesized by Genaxis Biotechnology (Nimes, France). Horseradish peroxidase-coupled sheep anti-mouse antibodies were from Jackson ImmunoResearch Laboratories. [5-<sup>14</sup>C]2-OG (2.07 GBq/mmol) and L-[<sup>35</sup>S]methionine (37 TBq/mmol) were purchased from Amersham Biosciences.

**Cell Lines and Culture Conditions**—C6 glioma cells were obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cells were maintained at 37 °C in 5% CO<sub>2</sub>, 95% air in a humidified incubator. Hypoxic incubations (2% O<sub>2</sub>) were performed using a CO<sub>2</sub> water incubator (Forma Scientific, Labtech, Model 3110).

**Reporter Plasmid Constructs**—The pHRE4-Luc plasmid (13) was kindly provided by Dr. Y. Fujii-Kuriyama. It was originally described as four tandemly repeated HRE motifs (5'-GATCGCCCTACGTGCTGTCTCA-3'). Sequence analysis indicated, however, that it formed a perfect palindromic structure that consists of four tandemly repeated HRE motifs followed by the complementary sequence inserted in the reverse orientation.

The pSV40-ODD-Luc vector containing the ODD domain of human HIF-1 $\alpha$  (GenBank<sup>TM</sup> accession number U22431) fused in-frame to the 5' end of the luciferase was prepared as follows. A 222-bp DNA fragment corresponding to amino acids 530–603 (nucleotides 1616–1837) of human HIF-1 $\alpha$  (pcDNA3-HIF-1 $\alpha$  vector kindly provided by Dr. P. J. Ratcliffe) was PCR-amplified using the sense primer 5'-CCGCTCGAGGCCACCATGGAATTCAAGTTGGAATTGGTA-3', corresponding to positions 1616–1636, and the reverse primer 5'-GCTCTAGACTGGAA-TACTGTAAC-3', corresponding to positions 1837–1823. The sequence includes the Pro-564 residue that is hydroxylated by prolyl hydroxylases (PHDs) (4, 5). The PCR product was digested by *Xba*I, blunted, and digested by *Xho*I. The resulting fragment was inserted into a *Xho*I/*Nco*I-blunted pSV40-Luc plasmid.

The pCMV-ODD-eGFP vector was prepared as follows. The same 222-bp PCR-amplified fragment of HIF-1 $\alpha$  was digested by *Xho*I and *Xba*I and fused in-frame with the eGFP of a *Xho*I/*Xba*I-digested pCI-eGFP2 plasmid (vector kindly provided by Dr. F. Lesage). The generated product, pCI-ODD-eGFP2, was then digested by *Bgl*II and *Not*I. The *Bgl*II-ODD-eGFP-*Not*I fragment was finally inserted into the *Bgl*II/*Not*I-digested pCDNA 3.1 (+) vector (Invitrogen).

The pCMV-eGFP vector was prepared as follows. The pCI-eGFP2 plasmid was digested by *Eco*RI and *Not*I. The *Eco*RI-eGFP-*Not*I fragment was purified and inserted into the *Eco*RI/*Not*I-digested pCDNA 3.1 (+) vector. Sequences of all products were confirmed by DNA sequencing using the dideoxy terminator method.

**Transient Transfections and Luciferase Assays**—Cells were transfected using LipofectAMINE (Invitrogen). They were incubated with a mixture of 10  $\mu$ g of plasmid DNA and 10  $\mu$ g of LipofectAMINE dissolved in 400  $\mu$ l of OPTI-MEM for 5 h at 37 °C. After a 16-h incubation time, cells were dissociated and seeded into 12-well tissue culture clusters. Eight h after seeding, cells were submitted to hypoxic conditions in the presence or absence of CsA as indicated. Luciferase assays (Promega) were performed according to the manufacturer's protocol. Luciferase activity was normalized by the total amount of cellular protein as assayed by the Bradford protein assay (Bio-Rad). Stable GFP and ODD-GFP cells were selected using 1.5 mg/ml G418 and cloned by limited dilution.

**Cell Lysis, Immunoblotting, and Immunoprecipitation**—Cells were washed twice in ice-cold PBS and lysed at 4 °C in lysis buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.5% Nonidet P-40, 1 mM sodium orthovanadate, 5 mM sodium fluoride, 50  $\mu$ M leupeptin, 50  $\mu$ g/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) for 20 min with continuous rocking and then centrifuged at 12,000  $\times$  g for 10 min. The supernatants were collected, and protein concentrations were determined by the Bradford protein assay. Protein extracts were electrophoresed and transferred to nitrocellulose membranes (Schleicher and Schuell) using standard procedures. For immunoprecipitation, lysis was performed in lysis buffer. After clearance by centrifugation, 250- $\mu$ g aliquots of cell lysate were incubated for 1 h at 4 °C with 5  $\mu$ g of anti-GFP monoclonal antibody followed by addition of protein A-Sepharose beads (10  $\mu$ l) and an overnight incubation. Immunoprecipitates were washed four times with lysis buffer, eluted with sample buffer, and immunoblotted. The membranes were blocked with 5% (w/v) instant non-fat milk powder in PBS, 0.1% Tween 20 for 1 h at room temperature followed by overnight incubation at 4 °C with the primary monoclonal antibodies against HIF-1 $\alpha$  (1:1,000), GFP (1:1,000), or  $\alpha$ -tu-

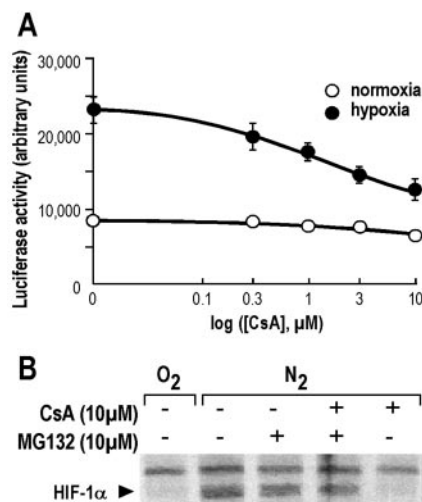
bulin (1:5,000). Monoclonal antibodies against GFP (1:1,000) or against vHL (1:2,000) were used to probe the immunoprecipitates. Blots were washed three times in PBS, 0.1% Tween 20, and incubated for 1 h at room temperature with horseradish peroxidase-conjugated sheep anti-mouse antibodies (1:10,000) in PBS, 0.1% Tween 20. After extensive washes in PBS, 0.1% Tween 20, chemiluminescence detection was performed by incubating the membranes with 100 mM Tris-HCl (pH 8.5), 2.65 mM H<sub>2</sub>O<sub>2</sub>, 0.45 mM luminol, and 0.625 mM coumaric acid for 1 min followed by exposure to x-ray films.

**HIF Peptide Hydroxylation Assay**—Rat kidneys were homogenized at 4 °C in 250 mM sucrose, 50 mM Tris-HCl (pH 7.5), 1  $\mu$ M leupeptin, 1  $\mu$ M bacitracin, and 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 1,000  $\times$  g for 10 min to remove cellular debris and nuclei. The supernatant was recovered and centrifuged at 3,000  $\times$  g for 10 min. The supernatant was recovered and centrifuged at 18,000  $\times$  g for 10 min. The pellet, usually referred to as the light mitochondrial fraction, was suspended in 40 mM Tris-HCl buffer at pH 7.5. Kidney extracts (0.3 mg of protein/ml) were incubated at 37 °C for 30 min in 40 mM Tris-HCl (pH 7.5), 0.5 mM dithiothreitol, 50  $\mu$ M ammonium ferrous sulfate, 1 mM ascorbate, 2 mg/ml bovine serum albumin, 0.4 mg/ml catalase, 50,000 dpm of [5-<sup>14</sup>C]2-OG, 0.1 mM unlabeled 2-OG, and 100  $\mu$ M of HIF peptide. HIF peptides used were: Pro-402 (DALTLAPAAAG-DTHSLDFK) and Pro-564 (LDLEALAPYIPADDDFQLRS), where the bold letters indicate Pro-402 and Pro-564. They corresponded to sequences 395–413 and 557–576 of human HIF-1 $\alpha$ . Methionine residues were converted to alanines to avoid spurious oxidation events. At the end of the reaction, the radioactivity associated to succinate was determined as described previously (14). The basal, peptide-independent, enzymatic activity was determined in each experiment. It represented less than 50% of total activity and was subtracted from all counts.

**Cloning, Mutagenesis, and Production of Glutathione S-transferase (GST)-ODD Fusion Proteins**—The 222-bp DNA fragment (nucleotides 1616–1837) of human HIF-1 $\alpha$  (pcDNA3-HIF-1 $\alpha$ ) was PCR-amplified with the forward 5'-GGAATTCAGTTGGAATTGG-3' and reverse 5'-CCGCTCGAGCTGGAATACTGTAAGTGT-3' set of primers. The P564A mutant was generated following three rounds of PCR amplification. (i) The 1616–1741 fragment was PCR-amplified with the forward 5'-GGAATTCAGTTGGAATTGG-3' and the reverse 5'-GTCATCATC-CATTGGGATATAGGCAGCTAACATCTCCAAGTCTAA-3' set of primers. (ii) The 1697–1837 fragment was amplified with the forward 5'-TTAGACTTGGAGATGTTAGCTGCCTATATCCCAATGGATGATG-AC-3' and the reverse 5'-CCGCTCGAGCTGGAATACTGTAAGTGT-3' set of primers. (iii) The final PCR amplification was initiated in the presence of an equimolar ratio of the two PCR-purified products and followed by the addition of the forward 5'-GGAATTCAGTTGGAATTGG-3' and the reverse 5'-CCGCTCGAGCTGGAATACTGTAAGTGT-3' primers. The ODD and the P564A ODD fragments were purified, digested with *Eco*RI and *Xho*I, and ligated into pGEX-4T-1 (Amersham Biosciences) to generate N-terminal GST-tagged fusion protein. Both plasmids were authenticated by DNA sequencing. The constructs (pGEX-GST-ODD and pGEX-GST-P564A) were transformed into *Escherichia coli* BL21(DE3), grown at 37 °C in LB media containing 100  $\mu$ g/ml ampicillin and induced with 0.1 mM isopropyl  $\beta$ -D-thiogalactoside for 4 h. Cells were harvested by centrifugation at 4,000  $\times$  g for 15 min and pelleted. Cells were sonicated on ice in lysis buffer (20 mM Tris-HCl (pH 7.5), 250 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 10% glycerol) supplemented with a mixture of protease inhibitors (Roche Molecular Biochemicals). After centrifugation at 100,000  $\times$  g for 30 min, the clarified sonicates were incubated with glutathione-Sepharose 4B (Amersham Biosciences) for 1 h at 4 °C. After four washes in PBS, the GST-tagged fusion proteins were eluted in 50 mM Tris-HCl, 10 mM reduced glutathione (pH 8). The integrity and yield of purified GST fusion proteins were assessed by SDS-PAGE followed by Coomassie Blue staining.

**GST Pull-down Assay**—Glutathione-purified GST-ODD and GST-ODD P564A fusion proteins were *in vitro* hydroxylated using a kidney homogenate prepared as described above. The reaction products were incubated at 4 °C in 200  $\mu$ l of buffer (50 mM Tris-HCl (pH 8), 120 mM NaCl, and 0.5% Nonidet P-40) supplemented with glutathione-Sepharose beads and 50,000 dpm of <sup>35</sup>S-labeled human vHL. After 2 h of incubation time, beads were washed three times with cold buffer (20 mM Tris-HCl (pH 8), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). The bound proteins were eluted in SDS running buffer and analyzed by SDS-PAGE followed by autoradiography. [<sup>35</sup>S]vHL was synthesized by using pcDNA3.1/V5-His-vHL as a template (a gift of Dr. S. L. McKnight) and the TnT-coupled reticulocyte lysate system (Promega).



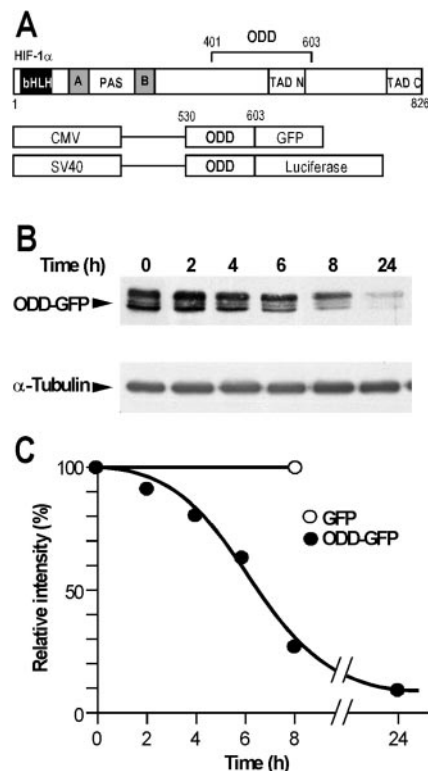


**FIG. 1. CsA inhibits HRE-mediated transcriptional activation and prevents hypoxia-induced accumulation of HIF-1 $\alpha$ .** *A*, dose-response curve for the inhibitory action of CsA on hypoxic response. Cells were transfected with pHRE4-Luc reporter plasmid and incubated under normoxic or hypoxic conditions for 16 h in the presence or absence of the indicated concentrations of CsA. This time of treatment corresponds to the peak of hypoxia-induced luciferase expression. Experiments were performed under hypoxic (filled symbols) or normoxic (open symbols) conditions. Luciferase expression was determined and normalized to protein content. Means  $\pm$  S.E. ( $n = 6$ ) are indicated. Essentially identical results were obtained in two other experiments. As shown in *B*, CsA inhibits the hypoxic expression of HIF-1 $\alpha$ . Cells were exposed to normoxic or hypoxic conditions for 4 h in the presence of 10  $\mu$ M CsA or 10  $\mu$ M MG132 as indicated. Whole cell extracts (80  $\mu$ g of protein) were resolved by SDS-PAGE and analyzed by immunoblotting using an anti-HIF-1 $\alpha$  antibody. The irrelevant cross-reacting band of higher molecular weight serves as internal and loading control.

## RESULTS

**CsA Inhibits HRE-mediated Transcription and Prevents the Hypoxic Stabilization of HIF-1 $\alpha$** —HRE-mediated transcriptional activation was analyzed using the pHRE4-Luc construct. The kinetics of transcriptional activation reveals that luciferase expression reached a 4-fold increase after 16 h of hypoxia (data not shown). Fig. 1*A* shows that hypoxia-induced luciferase expression was inhibited by CsA in a dose-dependent manner. In contrast, CsA did not alter reporter gene expression under normoxic conditions. Previous studies have demonstrated that HIF-1 $\alpha$  is rapidly degraded by ubiquitin-proteasome pathway under normoxic conditions (15). Activation of HIF-1 $\alpha$  to a functional form requires protein stabilization. Expression of HIF-1 $\alpha$  was analyzed using Western blots. Fig. 1*B* shows that a 4-h hypoxia induced a large accumulation of endogenous HIF-1 $\alpha$  protein. Stabilization of HIF-1 $\alpha$  was also observed under hypoxia in the presence of the proteasomal inhibitor MG132. CsA prevented most of the hypoxia-induced HIF-1 $\alpha$  stabilization, but in contrast, it was inactive in proteasomally blocked hypoxic cells. These data thus indicated that the inhibitory action of CsA requires a functional proteasomal machinery.

**CsA Abrogates ODD-mediated Protein Stabilization**—The ODD domain of HIF-1 $\alpha$  is responsible for the proteasomal degradation of HIF-1 $\alpha$  in normoxic cells. It has been located to the amino acid residues 401–603 of human HIF-1 $\alpha$ . This region overlaps with a vHL binding domain (526–641) (16, 17) and comprises two essential proline residues (Pro-402 and Pro-564) that are hydroxylated by PHDs under normoxic conditions (4, 5). To gain further insight into the mechanism of ODD-dependent protein stabilization, we generated chimeric proteins in which the C-terminal part of the ODD region of human HIF-1 $\alpha$  (residues 530–603) was fused to the GFP or luciferase open



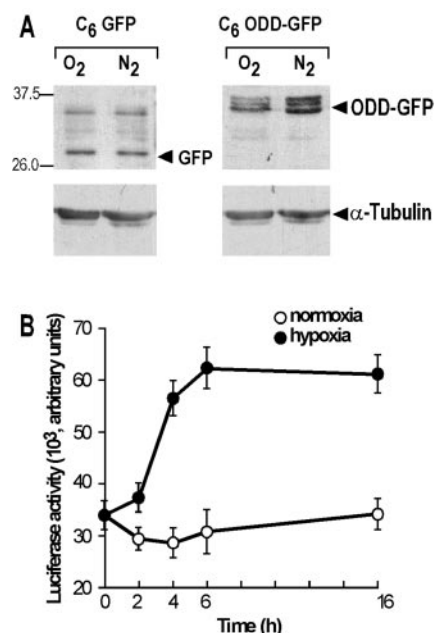
**FIG. 2. An ODD domain destabilized GFP.** *A*, schematic representations of HIF-1 $\alpha$ , pCMV-ODD-GFP, and pSV40-ODD-Luc constructs. Functional domains of HIF-1 $\alpha$  are indicated as follows: bHLH, basic-helix-loop-helix domain (residues 17–70); PAS, per-arnt-sim homology domain with internal A (residues 106–156) and B (residues 249–299) repeats; TAD-N (residues 531–575) and TAD-C (residues 786–826), N- and C-terminal transactivation domains; ODD, oxygen-dependent degradation domain (residues 401–603). CMV, cytomegalovirus. As shown in *B* and *C*, stable GFP or ODD-GFP transfectants were incubated in the presence of 100  $\mu$ g/ml cycloheximide to block protein synthesis. Expressions of GFP, ODD-GFP, or  $\alpha$ -tubulin were analyzed after different times using Western blots. *B*, representative blots showing the degradation of ODD-GFP. *C*, normalized ODD-GFP/ $\alpha$ -tubulin ratio. Means of triplicates are shown. Essentially identical results were obtained in four independent experiments.

reading frames (Fig. 2*A*). Stable transfectants expressing either ODD-GFP or control GFP were prepared. Expression of both proteins was analyzed by Western blots using an anti-GFP antibody. As expected, the antibody recognized a 27-kDa protein in GFP-expressing cells and a larger, 35–40-kDa, protein in ODD-GFP-expressing cells (Fig. 3*A*).

GFP is well known to be highly stable in mammalian cells with an estimated half-life of 24 h. Addition of an ODD domain to GFP was expected to destabilize the protein. The stability of ODD-GFP was determined after blocking protein synthesis with 100  $\mu$ g/ml cycloheximide. Fig. 2, *B* and *C*, shows that ODD-GFP was degraded with a half-life of about 6 h. The expression of the control GFP reporter was hardly affected after 8 h of exposure to cycloheximide. These results first confirmed the stability of GFP. They also indicated that the ODD sequence destabilized the GFP protein under normoxic conditions.

We next exposed the cells to hypoxia. Fig. 3*A* shows that hypoxia increased the expression of ODD-GFP but not that of GFP. The time course of hypoxia-induced protein expression is analyzed in Fig. 3*B* by using an ODD-luciferase reporter assay. Expression of luciferase reached a maximum after 4–6 h of hypoxia. An identical result was obtained with an ODD-GFP reporter or endogenous HIF-1 $\alpha$ .

Fig. 4*A* shows that CsA inhibited hypoxia-induced ODD-GFP



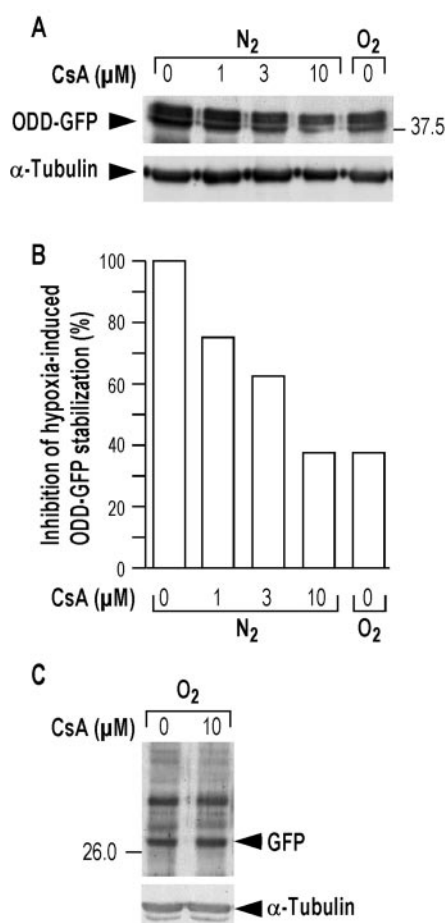
**FIG. 3. Hypoxia stabilizes ODD-GFP and ODD-Luciferase fusion proteins.** As shown in A, hypoxia stabilizes ODD-GFP but not GFP. ODD-GFP and GFP cells were incubated under normoxic or hypoxic conditions. Whole cell extracts (20  $\mu$ g of protein) were resolved by SDS-PAGE and immunoblotted with anti-GFP (top) or anti  $\alpha$ -tubulin (bottom) antibodies. B, time course of ODD-induced protein stabilization. Cells were transfected with a pSV40-ODD-Luc reporter plasmid. 24 h after transfection, cells were exposed to 2% O<sub>2</sub> (closed circles) or maintained at 21% O<sub>2</sub> (open circles) for the time period indicated. Results show normalized luciferase activities (Means  $\pm$  S.E.,  $n = 6$ ) and are representative of three independent experiments.

protein stabilization. The dose-response curve for the action of CsA is shown in Fig. 4B. CsA did not decrease expression of GFP (Fig. 4C). Thus, CsA required the presence of an ODD domain to be active. Note that the extent of the inhibitory effects of CsA on hypoxia-induced ODD-GFP fusion protein stabilization was comparable with those of HRE-mediated reporter gene transcription (Fig. 1B) and of hypoxia-induced HIF-1 $\alpha$  protein expression (Fig. 2B). All together, these observations indicated that CsA prevented the ODD-dependent stabilization of HIF-1 $\alpha$  in hypoxic cells.

**CsA Activates *In Vitro* Prolyl Hydroxylase Activity**—The oxygen-dependent destabilization of HIF-1 $\alpha$  is governed by a novel family of PHDs that specifically modify HIF-1 $\alpha$  at two conserved proline residues (Pro-402 and Pro-564) located in the ODD domain of HIF-1 $\alpha$ . One hypothesis for the previous results could be that CsA increased activity of PHDs. We therefore developed an assay for PHDs. Prolyl hydroxylations are mediated by 2-OG-dependent dioxygenases (18, 19) and can be followed by measuring the conversion of [5-<sup>14</sup>C]2-OG into [<sup>14</sup>C]succinate. Experiments were performed in the absence or presence of a HIF peptide that reproduced the 557–576 sequence of the ODD domain and that included Pro-564.

Experiments were first performed with extracts from normoxic cultured cells. We observed a degradation of 2-OG by cell extracts, but this activity was not stimulated by the HIF peptide (data not shown). It represents undefined enzymatic activities that are unrelated to PHDs. The absence of peptide-stimulated activity does not mean that PHDs were not present. It could be that the specific activity of 2-OG was too low to detect low levels of activity.

We then prepared homogenates from rat tissues and detected a large, peptide-dependent activity in whole kidney homogenates. The homogenate was fractionated by differential



**FIG. 4. CsA prevents the hypoxic stabilization of ODD-GFP fusion protein.** ODD-GFP (A and B) and GFP (C) cells were incubated under normoxic or hypoxic conditions in the presence or absence of the indicated concentrations of CsA. Whole cell extracts (20  $\mu$ g of protein) were resolved by SDS-PAGE and immunoblotted with anti-GFP (A) or anti  $\alpha$ -tubulin (C) antibodies. A, CsA inhibits hypoxia-induced stabilization of ODD-GFP. B, dose-response curve for the action of CsA using normalized ODD-GFP/ $\alpha$ -tubulin signal ratios. C, GFP expression is insensitive to CsA. Results shown are representative of 3–5 independent experiments.

centrifugations. Activity was recovered in a light mitochondrial fraction. The peptide-dependent activity did not require Fe(II) or ascorbate. It required 2-OG. The dose-response curve for 2-OG-induced activation of the enzyme is presented in Fig. 5A. Half-maximal activation was observed at 0.3 mM 2-OG. We also noticed that the peptide-independent enzymatic activity was more sensitive to 2-OG. As a consequence, large concentrations of 2-OG (0.3–1 mM) provide a better signal-to-noise ratio and should be used to monitor activity with precision.

Fig. 5B documents the influence of different concentrations of the Pro-564 peptide on the enzymatic activity. It also shows that CsA increased the degradation of 2-OG. CsA did not modify the basal, peptide-independent activity. The stimulating action of CsA was dose-dependent (Fig. 5C). Two other immunosuppressive drugs, FK506 and rapamycin, were inactive (data not shown).

We then performed experiments to demonstrate that 2-OG degradation was indeed associated to hydroxylation of Pro-564 by substituting the Pro-564 residue by a 4-hydroxyproline residue. This peptide was largely devoided of activity. We generated GST-ODD and GST-ODD P564A fusion proteins. GST-ODD promoted degradation of 2-OG to the same extent as the Pro-564 peptide (data not shown). The reaction products were probed with [<sup>35</sup>S]vHL. Fig. 5D shows that (i) degradation

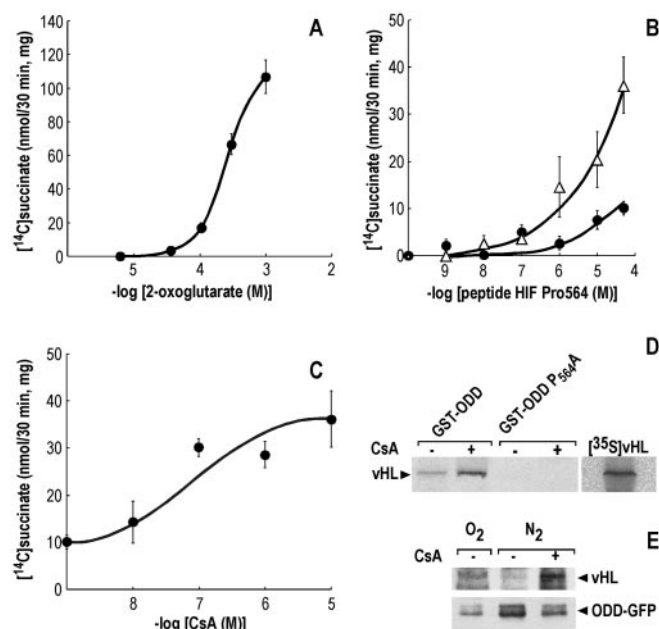


FIG. 5. **CsA activates Pro-564 hydroxylase.** A kidney light mitochondrial fraction was used as a source of enzyme. Activity was defined as the Pro-564 peptide-dependent degradation of 2-OG. The peptide-independent activity was determined in each experiment and was subtracted from the data. Means  $\pm$  S.E. from triplicate measurements are shown. **A**, 2-OG dependence. Experiments were performed in the presence of 100  $\mu\text{M}$  Pro-564 peptide. **B**, dependence on Pro-564 peptide. Experiments were performed in the absence (●) or the presence (▲) of 10  $\mu\text{M}$  CsA. **C**, dose-response curve for the stimulating action of CsA. Experiments were performed in the presence of 50  $\mu\text{M}$  Pro-564 peptide. **D**, vHL binding. GST-ODD (170  $\mu\text{g}$ ) was incubated for 30 min at 37  $^{\circ}\text{C}$  in the presence of the kidney extract and cofactors. Glutathione-Sepharose beads and 50,000 cpm of  $[^{35}\text{S}]\text{vHL}$  were then added. The bound vHL was recovered after 2 h at 4  $^{\circ}\text{C}$ , subjected to 15% SDS-PAGE, and visualized by phosphorimaging. The left lane shows a band from directly loaded  $[^{35}\text{S}]\text{vHL}$ . **E**, *in vivo* hydroxylation of ODD-GFP. ODD-GFP-expressing cells were incubated under normoxia or hypoxia in the presence or absence of 10  $\mu\text{M}$  CsA. ODD-GFP was immunoprecipitated by using an anti-GFP monoclonal antibody, resolved by SDS-PAGE, and probed with an anti-vHL (upper panel) or an anti-GFP (lower panel) monoclonal antibodies. Note that in this experiment, the expression of ODD-GFP was stabilized under hypoxia and reversed by addition of CsA. Results shown are representative of two other independent experiments.

of 2-OG was accompanied by an increased  $[^{35}\text{S}]\text{vHL}$  binding to ODD-GFP; (ii) CsA increased  $[^{35}\text{S}]\text{vHL}$  binding, as expected if it had stimulated PHD activity; and (iii)  $[^{35}\text{S}]\text{vHL}$  did not bind to GST-ODD P564A. Taken together, these results strongly support the hypothesis that CsA inhibited hypoxic responses by promoting hydroxylation of Pro-564 in the ODD domain.

Another substrate for PHDs is Pro-402. It is located in the N-terminal part of the ODD domain. The ODD domain used in the previous experiments comprised the 530–603 sequence of HIF-1 $\alpha$  and did not include Pro-402. We therefore synthesized a peptide that mimicked the 395–413 sequence of HIF-1 $\alpha$  and contains the Pro-402 residue. This peptide stimulated the degradation of 2-OG to the same extent as the Pro-564 peptide. Although we did not demonstrate hydroxylation of Pro-402, it would indicate that a light mitochondrial kidney fraction supported hydroxylations of both Pro-564 and Pro-402 peptides. Yet the Pro-402-dependent 2-OG degradation was insensitive to CsA. All together, our data suggest that CsA specifically promoted hydroxylation of Pro-564. Finally we tested poly(L-Pro-Gly-L-Pro), a well known substrate of procollagen prolyl hydroxylase(s). It was inactive in this assay.

**CsA Activates *In Vivo* Prolyl Hydroxylase Activity**—The previous results indicated that CsA increases *in vitro* PHD activity

when experiments were performed at ambient oxygen tensions. An obvious hypothesis for the inhibitory action of CsA on ODD-dependent protein stabilization is that CsA also increased PHD activity under conditions of reduced oxygen supply. To test this hypothesis, the ODD-GFP fusion protein was immunoprecipitated from normoxic or hypoxic cell extracts, and the bound, endogenous vHL was detected by Western blots using an anti-vHL antibody. Fig. 5E shows that hypoxia-stabilized ODD-GFP did not bind to vHL as expected since it was not hydroxylated. Addition of CsA destabilized ODD-GFP and increased vHL association. This is a direct demonstration that CsA promoted hydroxylation of ODD-GFP by activating Pro-564 PHD under hypoxic *in vivo* conditions.

## DISCUSSION

The mechanism by which hypoxia induces gene transcription involves the inhibition of HIF-1 $\alpha$  prolyl hydroxylase activity, which prevents vHL-dependent targeting of HIF-1 $\alpha$  to the ubiquitin-proteasome pathway. HIF-1 $\alpha$  is stabilized, associates with the aryl hydrocarbon receptor nuclear translocator, and interacts with HRE to promote the activation of target genes (see the Introduction). This study demonstrates that CsA inhibits the hypoxic signaling pathway by activating Pro-564 hydroxylation. (i) CsA prevents HRE-dependent gene expression in a dose-dependent manner. (ii) CsA blocks hypoxia-induced HIF-1 $\alpha$  protein accumulation, and this blockade is overcome by the proteasomal inhibitor MG132. (iii) CsA inhibits hypoxia-induced stabilization of an ODD-GFP chimeric protein, indicating that its action requires the C-terminal part of ODD. This sequence comprises the Pro-564 residue that is hydroxylated by PHDs and that is recognized by vHL (4, 5). (iv) CsA increases a kidney PHD activity that selectively hydroxylates Pro-564 in the ODD domain. (v) Finally, we present experimental evidence that CsA increases PHD activity in hypoxic cells. Inhibitors of PHDs have been developed (20) to promote hypoxic responses. CsA is the first known activator of a PHD activity. The major consequence of this stimulating action is an inhibition of hypoxic responses.

This report also describes an assay for PHDs. It was based on the capacity of peptides to stimulate the degradation of labeled 2-OG. Results indicated the presence in kidney extracts of enzymatic activities that were stimulated by Pro-402 and Pro-564 peptides. Both activities were enriched in light mitochondrial fractions and required exogenous 2-OG. They did not require Fe(II) or ascorbate, probably because cofactors were tightly bound to the enzymes. Based on our results, we cannot conclude that different PHDs mediate Pro-402 and Pro-564 hydroxylations. The same enzyme could mediate both reactions; however, only Pro-564 hydroxylation is activated by CsA. Four different PHDs are now identified (18, 19, 21). The identity of the enzyme(s) present in our light mitochondrial kidney fractions is not known. However, it is of interest to note that SM-20 contains a mitochondrial targeting sequence (22) and that the newly described PHD-4 isoform is highly expressed in kidney tissues and associates to light microsomes (21).

CsA is a well known immunosuppressive drug that binds to cyclophilins, a family of ubiquitous and conserved proteins with peptidyl-prolyl *cis-trans* isomerase and molecular chaperone activities (23, 24). We now show, for the first time, that CsA specifically stimulates Pro-564 hydroxylation. A hypothesis could be that hydroxylation of Pro-564 requires a specific configuration of the proline residue and that a cyclophilin controls in some way the presentation of the peptide to the enzyme. This hypothesis is unlikely, however, since CsA should not be active in hypoxic cells in which PHD activity is limited by oxygen availability. Indeed, in agreement with this observation, we were unable to show by immunoprecipitation experiments, us-



ing whole cell and kidney protein extracts, a direct interaction of cyclophilins (Cyp A and Cyp 40) with ODD substrates (GST-ODD, ODD-GFP, or endogenous HIF-1 $\alpha$ ). Another hypothesis could be that a cyclophilin associates to one PHD and controls its oxygen sensitivity. This hypothesis would agree with the recent evidence suggesting the existence of more than one oxygen sensor (18).

Rapamycin and FK506 recognize and bind to another class of peptidyl-prolyl isomerases, termed FK-binding proteins (FKBP). Rapamycin, like CsA, inhibits cellular responses to hypoxic stresses by destabilizing the ODD domain of HIF-1 $\alpha$  (25). Yet the molecular mechanisms involved are different. CsA promotes hydroxylation of the Pro-564 peptides. Rapamycin and FK506 do not. Hudson *et al.* (25) further provided evidence that rapamycin acts via mammalian target of rapamycin, a downstream target of the phosphatidylinositol 3-kinase. All together, these findings further support the idea that HIF-1 $\alpha$  protein stabilization can be achieved by PHD-dependent and -independent mechanisms (26).

CsA induces renal and systemic vasoconstrictions that are consecutive to changes in the production of vasoactive substances, such as endothelin-1 and nitric oxide. This study shows that CsA prevents HRE-mediated hypoxic adaptation. It is important to note that such a mechanism would exacerbate the toxicity of hypoxia that results from the vasoconstriction and may well contribute to the severity of hypoxic nephropathies. It is of interest to note that FK506, which is less nephrotoxic than CsA, does not promote PHD activity in kidney microsomes.

In conclusion, this report shows that CsA increases peptidyl-prolyl hydroxylase activity in kidney extracts and hypoxic cells. By this mechanism, CsA abrogates hypoxic stabilization of HIF-1 $\alpha$  and HIF-1 $\alpha$ -mediated cellular responses in glioma cells. CsA can thus be considered as an oxygen sensitizer that limits adaptative responses to hypoxia.

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