**Communication**

**p53 Inhibits Hypoxia-inducible Factor-stimulated Transcription***

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p53 is required for hypoxia-induced apoptosis in *vivo*, although the mechanism by which this occurs is not known. Conversely, induction of the hypoxia-inducible factor-1 (HIF-1) transactivator stimulates transcription of a number of genes crucial to survival of the hypoxic state. Here we demonstrate that p53 represses HIF-1-stimulated transcription. Although higher levels of p53 are required to inhibit HIF than are necessary to transcriptionally activate p53 target genes, these levels of p53 are similar to those that stimulate cleavage of poly-(ADP-ribose) polymerase, an early event in apoptosis. Transfection of full-length p300 stimulates both p53-dependent and HIF-dependent transcription but does not relieve p53-mediated inhibition of HIF. In contrast, a p300 fragment, which binds to p53 but not to HIF-1, prevents p53-dependent repression of HIF activity. Transcriptionally inactive p53, mutated in its DNA binding domain, retains the ability to block HIF transactivating activity, whereas a transcriptionally inactive double point mutant defective for p300 binding does not inhibit HIF. Finally, depletion of doxorubicin-induced endogenous p53 by E6 protein attenuates doxorubicin-stimulated inhibition of HIF, suggesting that a p53 level sufficient for HIF inhibition can be achieved in vivo. These data support a model in which stoichiometric binding of p53 to a HIF/p300 transcriptional complex mediates inhibition of HIF activity.

The p53 tumor suppressor protein mediates both growth arrest and apoptosis. Whereas p53-dependent growth arrest requires transcriptional activation of p21WAF1/CIP1 (1–5), substantial data have accumulated that transcriptionally capable is not necessary for p53 to stimulate apoptosis (4–6).

Hypoxia is perhaps the most physiologic inducer of p53 (7), and hypoxia-mediated apoptosis of *tumors in vivo* requires p53 (8). Indeed, p53 is the most frequently inactivated gene in solid tumors, and in animal tumor models, hypoxia selects against wild type p53 (8). However, the mechanism by which p53 mediates apoptosis in hypoxic tumor cells is not known. Hypoxic conditions in *vitr* as well as in *vivo* result in induction of hypoxia-inducible factor-1α (HIF-1α),1 the limiting, hypoxia-inducible subunit of the HIF-1 transactivator (9). HIF-1, in turn, stimulates transcription of a number of genes important for tumor survival under hypoxic conditions in *vivo*, including vascular endothelial growth factor (VEGF), erythropoietin (Epo), and several glycolytic enzymes (10). Tumors in which hypoxia cannot induce HIF-1 transcriptional activity remain small and fail to metastasize (11).

We have recently shown that hypoxic induction of p53 requires concomitant induction of HIF-1α, and that HIF-1α binds to and stabilizes p53 (12). We found previously that HIF-1α had no direct effect on p53 transcriptional activity. We now report that association of HIF-1α and p53 results in inhibition of HIF-stimulated transcription. This requires a higher p53 level than is necessary for transcriptional activation of several endogenous p53-responsive promoters but correlates well with the level of p53 necessary to cause apoptosis.

**MATERIALS AND METHODS**

*Cell Lines—* SKBr3 and MCF7 are human breast cancer cell lines obtained from American Type Culture Collection (Rockville, MD), and PC3M is a highly metastatic variant of the prostate cancer cell line. PC3. SKBr3 contains one mutated, transcriptionally inactive p53 allele, MCF7 contains transcriptionally active wild type p53 (wtp53), and PC3M cells are p53-null. Reagents and Plasmids—Ad-LacZ, a β-galactosidase-expressing, replication-deficient adenovirus, and Ad-p53, a wtp53-expressing, replication-deficient adenovirus, were obtained from B. Vogelstein (Johns Hopkins Oncology Center). Viral titer was determined as described previously (13). Multiplicity of infection (MOI) is defined as the ratio of total number of viruses used in a particular infection per number of cancer cells to be infected (i.e., number of viruses per cell).

Plasmids WWP-Luc, a p21 promoter-luciferase construct, PG13-Luc, containing a generic p53 response element, pCMVβ wt-p53, and the p53 mutant p53–273H were obtained from B. Vogelstein. Bax-Luc, a Bax promoter-luciferase construct and the p53 double point mutant p53(22/23) were obtained from K. Vousden (ABL Basic Research Program, NCI-FCRDC). The pCMVβHA-HIF-1α expression plasmid was obtained from D. Livingstone (Dana Farber Cancer Institute). An HIF-responsive, VEGF promoter-derivered luciferase construct containing four amplified HIF-1 binding sites (VEGF-Luc), inserted into a pGL2-promoter vector was obtained from A. J. Giaccia (Stanford University) and described previously (14). An HIF-responsive, erythropoietin promoter-derived luciferase construct (Epo-Luc) and its HIF-insensitive mutant variant (mEpo-Luc), inserted into a pGL3-Promoter vector were obtained from F. Bunn and E. Huang (Harvard Medical School). The control luciferase plasmid, pGL2-control, driven by SV40 promoter and enhancer sequences, was purchased from Promega Corp. (Madison, WI). The full-length p300 expression plasmid, pCMVβ p300, and the p53-binding p300 fragment, pCMVβ p300 1514–1922, were obtained from K. Vousden (NCI). pCMV β-galactosidase was purchased from CLONTECH (Palo Alto, CA). pCMV neo16E6 was obtained from K. Vousden.

**Transient Transfection Assay—** 8 × 104 or 4 × 104 cells were plated in T25 flasks or 6-well plates (Costar, Acton, MA), respectively. The next day, cells were transfected with plasmids in the presence of LipofectAMINE (Life Technologies, Inc.). After 6–12 h of incubation with the plasmid-lipid suspension, the medium was changed, and cells were grown for an additional 24 h, unless otherwise indicated. The cells were lysed and analyzed for luciferase activity as described previously (12).

**Western Blot Analysis—** Proteins were resolved with 8% SDS-poly-
acrylamide gel electrophoresis for detection of p53, Mdm-2, and poly-(ADP-ribose) polymerase (PARP) as described previously (15).

DNA Synthesis—DNA synthesis was monitored by [3H]thymidine incorporation (13).

RESULTS AND DISCUSSION

Wild Type p53 Abrogates HIF-1α Activity—We initially investigated the effects of wt p53 on HIF-1α-responsive transcription in SKBr3 and PC3M cell lines. Transient expression of HIF-1α dramatically induced transcription of two HIF-dependent reporter constructs, Eco-Luc (containing an HIF-responsive element from the erythropoietin gene) and VEGF-Luc (containing HIF-responsive elements from the VEGF gene) in both cell lines, whereas co-transfection of wt p53 abrogated this induction (Fig. 1, A, B, and D). Dose-response analysis showed near-maximal inhibition of HIF-1α-stimulated transcription (using 0.5 μg of HIF-1α) by as little as 0.1 μg of wt p53 in SKBr3 cells and 0.5 μg of wt p53 in PC3M cells (Fig. 1, A and B).

The Transactivating Function of p53 Is Not Required for Suppression of HIF-1α Activity—In order to determine whether transactivating capability was essential for p53-mediated HIF inhibition, we next examined the HIF-1α inhibitory activity of the p53 “DNA contact” mutant p53–273H (16), which failed to transactivate three independent p53-responsive reporters: PG13-Luc, WWP-Luc, and Bax-Luc (data not shown). Although lacking transactivating capability, p53–273H repressed HIF-1α-driven transcription in both SKBr3 and PC3M cells (Fig. 1, A, B, and D), demonstrating that neither DNA binding nor transactivation is essential for p53-mediated suppression of HIF-1α activity.

To confirm the specificity of this phenomenon, we tested the effects of both wt p53 and p53–273H on a control luciferase plasmid, pGL2-control (VEGF-Luc is a pGL2-based reporter and Eco-Luc is a pGL3-based reporter), in transiently transfected PC3M cells. At the concentrations used in these experiments, neither p53 construct significantly inhibited the SV40-driven luciferase activity generated from pGL2-control-transfected cells (Fig. 1C).

p53-mediated HIF Suppression Correlates with Stimulation of PARP Cleavage but Not with the Transactivating Function of p53—Although p53-mediated suppression of HIF activity can be clearly demonstrated, it requires higher p53 levels than are necessary to observe p53-mediated transactivation. Thus, transactivation of PG13-Luc in SKBr3 cells requires co-transfection of 10–20-fold less p53 than is necessary to obtain significant HIF-1α inhibition (Fig. 2A). Because transfection does not introduce p53 into 100% of the cells tested, it is difficult to compare suppression of HIF activity with the growth inhibitory and apoptotic activity of p53. Since we demonstrated previously that essentially 100% of cells infected with the p53-containing adenovirus Ad-p53 express wt p53 protein following infection (minimum MOI = 2; see Ref. 13), we used this approach to further explore the relationship of p53 expression to transactivation and HIF suppression, respectively.

We determined the MOI of Ad-p53 required in SKBr3 cells to yield maximal inhibition of Eco-Luc (Fig. 2B) and DNA synthesis (Fig. 2C), stimulation of PARP cleavage (Fig. 2D), induction of Mdm-2 protein (Fig. 2E), and stimulation of Bax-Luc (Fig. 2F). To control for any nonspecific effects of viral titer, cells were infected with 32 MOI of Ad-LacZ (second bar/lane in Fig. 2, B–F). PARP cleavage is an early event in apoptosis, and inhibition of DNA synthesis is a marker of growth arrest. The data demonstrate that near-maximal inhibition of DNA synthesis, Mdm-2 induction, and transactivation of Bax-Luc occur at an Ad-p53 MOI of 2–4. In contrast, both significant inhibition of HIF-responsive transcription and stimulation of PARP cleavage do not occur until an MOI of 16–32 is reached. Infection with Ad-LacZ was consistently without effect. Thus, whereas the transactivating function of p53 correlates with induction of growth arrest, suppression of HIF activity correlates with the higher amount of p53 required to initiate apo-
cells infected with 32 MOI of Ad-LacZ. The genes in
fection. In B
stimulation of Bax-Luc (A) and fold inhibition of VEGF-Luc are shown on the
left axis. Luciferase activity was determined as in Fig. 1. Fold stimulation of PG13-Luc and fold inhibition of VEGF-Luc are shown on the left and right axes, respectively. B–F, SKBr3 cells were infected with an increasing MOI of Ad-p53, and inhibition of Epo-Luc (B), inhibition of DNA synthesis (C), stimulation of PARP cleavage (D), induction of Mdm-2 protein (E), and stimulation of Bax-Luc (F) were determined. In each case, the first bar or gel lane depicts uninfected cells, and the second bar or lane (32×) depicts cells infected with 32 MOI of Ad-LacZ. The numbers under the remaining bars (with gel lanes corresponding) refer to the MOI of Ad-p53 used for infection. In B, all cells were co-transfected with Epo-Luc (0.5 μg) and CMV.HA-HIF-1α (0.5 μg). In F, all cells were co-transfected with Bax-Luc (0.5 μg). In B and F, transfections were performed 12–16 h prior to viral infection, and assays were performed 24 h following viral infection. In C–E, analyses were performed 24 h following viral infection.

ptosis. Similar to what was found in the previous transfection experiment (Fig. 2A), 8–16-fold more p53 was needed to suppress HIF activity and stimulate PARP cleavage than was required to support transactivation of p53 target genes and to cause growth arrest.

These results are in agreement with our earlier observation that transactivation does not play a role in the inhibition of HIF activity by p53. Taken together with our recent observation that p53 co-precipitates with HIF-1α (12), the data fit a model in which direct association of HIF-1α with p53 results in HIF inhibition.

p300 Interaction with p300 Is Required for Inhibition of HIF Activity—Both HIF-1α and p53 bind to p300, and p300 is required for full activity of both transactivators (17–21). Thus, it was of interest to determine whether p300 plays any role in p53-mediated HIF inhibition. We first determined whether exogenous p300 could restore HIF activity in the presence of p53. We transfected PC3M cells with Epo-Luc and HIF-1α, plus either CMV-β-galactosidase (control plasmid) or wtp53, and full-length p300 (Fig. 2A). Although exogenous p300 augmented HIF-dependent transcriptional activity, as reported previously (17), it could not reverse p53-mediated inhibition of this activity.

HIF-1α and p53 bind to distinct regions of p300. The HIF binding site has been localized to a region encompassed by amino acids 346–410 (17), whereas p53 has been reported to bind to a p300 fragment comprising amino acids 1514–1922 (18, 21). These findings raise the possibility that p53 and HIF-1α can bind to p300 simultaneously, leading to interference by p53 in HIF/p300-mediated transactivation. This model predicts that exogenous p300 would be unable to reverse HIF activity an additional 5-fold compared with transfection with HIF-1α alone, whereas Epo-Luc activity in cells co-transfected with p300(1514–1922) and HIF-1α was 0.6-fold greater than in cells transfected with HIF-1α alone. B, PC3M cells were transfected with either wtp53 (0.25 μg) or the double point mutant p53(22/23) (2 μg), together with Epo-Luc (0.5 μg) and HA-HIF-1α (0.5 μg). Luciferase activity was determined after 36 h.

FIG. 2. p53-stimulated suppression of HIF activity correlates with PARP cleavage but not with p53-mediated transactivation or growth arrest. A, comparison of the amounts of pcMV.wtp53 necessary to stimulate PG13-Luc and repress VEGF-Luc. SKBr3 cells were transfected with PG13-Luc (0.5 μg, open circles) or VEGF-Luc (0.5 μg) together with CMV.HA-HIF-1α (0.5 μg, closed circles) and co-transfected with increasing amounts of CMV.wtp53 as indicated on the x axis. Luciferase activity was determined as in Fig. 1. Fold stimulation of PG13-Luc and fold inhibition of VEGF-Luc are shown on the left and right axes, respectively. B–F, SKBr3 cells were infected with an increasing MOI of Ad-p53, and inhibition of Epo-Luc (B), inhibition of DNA synthesis (C), stimulation of PARP cleavage (D), induction of Mdm-2 protein (E), and stimulation of Bax-Luc (F) were determined. In each case, the first bar or gel lane depicts uninfected cells, and the second bar or lane (32×) depicts cells infected with 32 MOI of Ad-LacZ. The numbers under the remaining bars (with gel lanes corresponding) refer to the MOI of Ad-p53 used for infection. In B, all cells were co-transfected with Epo-Luc (0.5 μg) and CMV.HA-HIF-1α (0.5 μg). In F, all cells were co-transfected with Bax-Luc (0.5 μg). In B and F, transfections were performed 12–16 h prior to viral infection, and assays were performed 24 h following viral infection. In C–E, analyses were performed 24 h following viral infection.

FIG. 3. p53 interaction with p300 is necessary for repression of HIF-1 activity. A, PC3M cells were transfected with Epo-Luc (0.5 μg) and CMV.HA-HIF-1α (0.5 μg) and cotransfected with CMV.β-galactosidase (0.2 μg, lanes 1, 3, and 5), CMV.wtp53 (0.2 μg, lanes 2, 4, and 6), PCMV.p300 (2.0 μg, lanes 3–4), and PCMV.p300(1514–1922) (2 μg, lanes 5–6). Luciferase activity and Mdm-2 protein levels were determined after 36 h. For each pair of bars, the value obtained in the absence of transfected p53 was set to 100%. In the absence of p53, co-transfection of HIF-1α and full-length p300 augmented Epo-Luc activity an additional 5-fold compared with transfection with HIF-1α alone, whereas Epo-Luc activity in cells co-transfected with p300(1514–1922) and HIF-1α was 0.6-fold greater than in cells transfected with HIF-1α alone. B, PC3M cells were transfected with either wtp53 (0.25 μg) or the double point mutant p53(22/23) (2 μg), together with Epo-Luc (0.5 μg) and HA-HIF-1α (0.5 μg). Luciferase activity was determined after 36 h.
suppression as long as intracellular p53 levels exceed HIF levels. Such a stoichiometric hypothesis can also explain why significantly higher amounts of p53 are necessary to inhibit HIF than are required to transactivate target genes and why p53 need not be transactivation competent to mediate HIF suppression.

Based on this model, a p300 fragment that bound p53 but not HIF-1α would be expected to block p53-mediated HIF repression. To test this, we repeated the above experiment but co-transfected with the p53-binding p300 fragment, p300(1514–1922), instead of full-length p300. Since it lacks the HIF-1α binding site this fragment did not further stimulate HIF activity, but it markedly ameliorated p53-mediated inhibition of this activity (Fig. 3A). Co-transfection of p300(1514–1922) did not affect the level of expression of p53 (not shown), but it did abrogate the ability of p53 to induce endogenous Mdm-2 (see Fig. 3A, inset), previously shown to be dependent on p53/p300 interaction (21). These data suggest that p300(1514–1922) reversed p53-mediated inhibition of HIF-dependent transcription by sequestering p53 and preventing its association with the endogenous pool of p300.

In order to further explore the requirement for p300 in p53-mediated inhibition of HIF-1, we tested the anti-HIF activity of a p53 double point mutant, p53(22/23), which is mutated at residues 22 and 23 in the amino-terminal transactivating domain (5, 22) and does not bind to p300 (21). As can be seen in Fig. 3B, this mutant was unable to suppress HIF-1 activity in PC3M cells. Although 0.25 μg of wt p53 markedly inhibited HIF-1, p53(22/23) was inactive even when transfected at 2 μg. Both wt p53 and p53(22/23) were expressed to similar levels, as monitored by Western blotting (not shown). Taken together, these data demonstrate that p53 must bind to p300 in order to inhibit HIF-1.

Drug-induced Elevation of Endogenous wt p53 Suppresses HIF-1 Activity—Although exogenously supplied p53 inhibits HIF-1-driven transcription at levels that initiate apoptotic events, we wished to determine whether endogenous p53 could be induced to display similar activity. Thus, we transfected MCF7 cells, which contain wt p53, with Epo-Luc and HIF-1α and examined the effect on reporter activity of the DNA-damaging drug doxorubicin, a potent inducer of wt p53. Treatment with doxorubicin led to marked elevation of p53 and significantly blunted HIF transcriptional activity (Fig. 4). Co-transfection of a human papilloma virus E6-expressing plasmid together with Epo-Luc and HIF-1α reversed this inhibition (Fig. 4). In contrast, doxorubicin had no effect on HIF-1 activity in p53-null PC3M cells (data not shown).

In summary, we have demonstrated that p53 is able to repress HIF activity in a manner not requiring the transactivating function of p53. That a significantly greater amount of p53 is necessary to inhibit HIF than is required to transactivate p53 target genes or cause growth arrest can be explained by a stoichiometric model in which p53 must saturably bind to HIF/p300-containing complexes in order to inhibit HIF. The physiologic relevance of this phenomenon is supported by two observations. First, the level of p53, which must be reached to mediate HIF repression, is equivalent to that at which PARP cleavage becomes detectable, and second, this level of p53 can be reached in vivo following exposure to a commonly used chemotherapeutic.

REFERENCES

1. Brugge, J., Chandrasekaran, C., Gordon, J. I., Beach, D., Jacks, T., and Hannon, G. J. (1995) Nature 377, 552–557
2. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J., and Leder, P. (1995) Cell 82, 675–684
3. Waldman, T., Kindler, K. W., and Vogelstein, B. (1995) Cancer Res. 55, 5187–5190
4. Caillé, C., Heilberg, A., and Karin, M. (1994) Nature 370, 220–223
5. Haupt, Y., Rowan, S., Shaulian, E., Vousden, K. H., and Oren, M. (1995) Genes Dev. 9, 2170–2183
6. Rowan, S., Ludwig, R., Haupt, Y., Bates, S., Lu, X., Oren, M., and Vousden, K. H. (1996) EMBO J. 15, 827–838
7. Graeber, T. G., Peterson, J. F., Tsai, M., Monica, K., Fornace, A. J., Jr., and Giaccia, A. J. (1994) Mol. Cell. Biol. 14, 6264–6277
8. Graeber, T. G., Osmanian, C., Jacks, T., Housman, D. E., Koch, C. J., and Giaccia, A. J. (1996) Nature 379, 88–91
9. Wang, G. L., Jiang, B.-H., Rue, E. A., and Semenza, G. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5510–5514
10. Guillenin, K., and Krasnow, M. A. (1997) Cell 89, 9–12
11. Maxwell, P. H., Dachs, G. U., Gleadle, J. M., Nicholls, L. G., Harris, A. L., Stratford, I. J., Hanksinson, O., Pugh, C. W., and Ratcliffe, P. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8104–8109
12. An, W. G., Knebak, M., Simon, M. C., Maltepe, E., Blagosklonny, M. V., and Neckers, L. M. (1998) Nature 392, 405–408
13. Blagosklonny, M. V., and El-Deiry, W. S. (1996) Int. J. Cancer 67, 386–392
14. Mazure, N. M., Chen, E. Y., Yeh, P., Larderoute, K. R., and Giaccia, A. J. (1996) Cancer Res. 56, 3436–3440
15. Blagosklonny, M. V., Toretsky, J., Bohe, S., and Neckers, L. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8579–8583
16. Rolley, N., Butcher, S., and Milner, J. (1995) Oncogene 11, 763–770
17. Arany, Z., Huang, L. E., Eckner, R., Bhattacharya, S., Jiang, C., Goldberg, M. A., Bunn, H. F., and Livingston, D. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12969–12973
18. Avantaggiati, M. L., Ogryzko, V., Gardner, K., Giordano, A., Levine, A. S., and Kornberg, A. D. (1996) Cell 85, 88–91
19. Lill, N. L., Grossman, S. R., Ginsberg, D., DeCaprio, J., and Livingston, D. M. (1996) Nature 387, 823–827
20. Somasundaram, K., and El-Deiry, W. S. (1997) Oncogene 14, 1047–1057
21. Gu, W., Shi, X.-L., and Roeder, R. G. (1997) Nature 387, 819–823
22. Lin, J. Y., Chen, J. D., Elenbaas, B., and Levine, A. J. (1994) Genes Dev. 8, 1235–1246