Tumor Suppressor p53 Is a Negative Regulator in Thyroid Hormone Receptor Signaling Pathways*

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* The abbreviations used are: T3, 3,3′,5-triiodo-L-thyronine; TR, thyroid hormone nuclear receptor; h-TRβ1, human TR subtype β1; TRE, thyroid hormone response element; Pal, a palindromic TRE; Lys, the transcription factor that plays a critical role in cell cycle regulation and tumor development. The molecular mechanisms by which TRs mediate these diverse effects are unclear. One emerging hypothesis suggests that TRs could mediate these diverse effects via cooperation with other transcription factors/receptors. Indeed, we have recently shown that the human TR subtype β1 (h-TRβ1) interacts with the tumor suppressor p53. p53 is a transcription factor that plays a critical role in cell cycle regulation and tumor development. To assess the physiological relevance of the interaction of h-TRβ1 with p53, the present study addressed the question as to whether the functions of h-TRβ1 could be modulated by p53. We first compared the h-TRβ1-mediated transcriptional activity in two pairs of isogenic cell lines, RKO/KO E6 and MCF-7/MCF-7 E6. RKO and MCF-7 cells are colon and breast carcinoma cell lines, respectively, that contain p53 but lack TRβ1. The isogenic RKO E6 and MCF-7 E6 cells are stable clones expressing high levels of papillomavirus type 16 E6 protein. In these cells, the level of p53 protein was lower than the parental cells. The impairment of p53 functions in these E6-containing cells led to an activation of TRβ1-mediated transcriptional activity. Furthermore, in a growth hormone-producing cell line in which the expression of the growth hormone gene is positively regulated by TRs, overexpression of the wild-type p53 led to repression in the expression of the growth hormone gene. Thus, TRs could cross-talk with p53 in its signaling pathways to regulate gene regulatory functions. The present findings further strengthen the hypothesis that mediation of the pleiotropic effects of T3 requires the cooperation of TRs with a large network of transcription factors.

The thyroid hormone 3,3′,5′-triiodo-L-thyronine (T3) promotes growth, induces differentiation, and regulates metabolic functions. These effects are mediated by the interaction of T3 with the thyroid hormone nuclear receptors (TRs). Thyroid hormone receptors (TRs) belong to the steroid hormone/retinoic acid receptor superfamily and function as ligand-dependent transcription factors. Two TR genes, α and β, encode two receptor variants through alternative splicing of each of the primary transcripts. The gene regulating activity of TRs depends not only on T3 but also on the specific DNA sequences in the promoter regions of T3 responsive genes, known as the thyroid hormone response elements (TREs) (1). Recent studies have indicated that the gene regulating activity of TR is further modulated via interaction with other cellular proteins including several members of the nuclear receptor superfamily (1–7).

Despite recent progress, the molecular mechanisms by which TRs mediate the T3 biological activities are still unclear. One of the central issues is how the diverse effects of T3 are achieved. We hypothesized that the diverse effects of T3 could be mediated by interaction of TRs with other transcription factors/cellular factors in the signaling pathways of TRs. Thus, the hormone signal mediated by TRs could be modulated by the TR-interacting transcription factors depending on the cellular context. Recently, we searched for such transcription factors and found that the tumor suppressor p53 physically interacts with human TR subtype β1 (h-TRβ1) (8). p53 is a transcription factor that plays a critical role in cell cycle regulation and tumorigenesis. It activates transcription by binding to specific DNA sequences known as p53 response elements (9, 10). However, it can also repress expression of genes that lack p53 response elements (11–13). In addition, p53 interacts avidly with a variety of cellular proteins and viral proteins leading to modifications of the biochemical activities and/or functions of these associated proteins (13).

We have previously shown that the physical interaction of h-TRβ1 with p53 leads to the inhibition of the binding of h-TRβ1 to TREs in a concentration-dependent manner (8). Using exogenous genes transfected into TR-deficient cells, we found that T3-dependent h-TRβ1-mediated transactivation activity is inhibited by p53 (8). However, the functional relevance of this interaction has not been defined (8). The present study adopted two independent systems to address the question of whether the interaction of p53 with TRs led to functional consequences. In one system, we used two pairs of isogenic cell lines in which the expression of endogenous p53 differed so that the effect of p53 on the transcriptional activity of h-TRβ1 can be compared within a pair. In another system, we utilized a clonal growth hormone-producing cell line, GC cells, in which the expression of the growth hormone is under the control of TRs (14–17). The effect of p53 on the function of TRs can be conveniently assessed by the alterations in the expression of...
the growth hormone gene. Using these two systems, we found that the function of TRs was modulated by p53, indicating that p53 interacted with the TR-mediated signaling pathways.

MATERIALS AND METHODS

3-3,5,3'-dichloroacetetyl-1,4-6-Chloramphenicol (2.04 GBq/mmol; 55 mCi/mmole) (1 Ci = 37 GBq) was obtained from NEN Life Science Products. An ECL Western blotting kit was obtained from Amersham (Buckinghamshire, UK). Lipofectamine reagent and Opti-MEM I reduced serum medium were purchased from Life Technologies, Inc. The stable breast carcinoma MCF-7 and colon carcinoma RKO clones containing human papillomavirus type 16 E6 gene (MCF-7 E6 and RKO E6) and their isogenic pairs containing only the vector (MCF-7 and RKO) were generously provided by Albert J. Fornace (NCI, Bethesda, MD) (18). They were cultured in DMEM containing 10% fetal bovine serum (Life Technologies, Inc.) (19). Stable breast carcinoma MCF-7 and colon carcinoma RKO clones containing human papillomavirus type 16 E6 gene were grown in DMEM containing thyroid hormone-depleted calf serum (Life Technologies, Inc.) (20).

Detection of p53 by Western Blotting—Isogenic pairs of RKO/RKO E6 and MCF-7/MCF-7 E6 cells (4 × 10^6 cells/15-cm dish) were cultured for 48 h and treated with or without irradiation using a 137Cs source delivering γ-rays at a dose rate of 3.46 Gy/min. After irradiation, the media in all the plates were replaced with fresh media, and the cells were further cultured for 4 h at 37 °C. Cells were harvested, washed twice with cold phosphate-buffered saline, and lysed on ice for 1 h in 1% Nonidet P-40, 50 mM NaCl, 50 mM Tris-HCl, pH 7.5. The lysates were analyzed by SDS-PAGE, and the Western blotting was carried out as described above using anti-p53 antibodies as described under “Materials and Methods,” the protein bands were visualized by autoradiography.

Analysis of p53 Protein Expression in Ad-p53-infected GC Cells—Western blotting was used to analyze the expression of p53 in GC cells after infection with Ad-p53. GC cells (1.5 × 10^6/10-cm dish) were infected as described above. Cells were lysed in RIPA buffer (0.5% SDS, 1% Nonidet P-40, 0.1% sodium deoxycholic acid, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5). The lysates were analyzed by SDS-PAGE, and Western blotting was carried out as described above using anti-p53 antibodies to determine the functional consequences of the interaction of h-TRβ1 with p53. These two cell lines contain the wild-type p53 but lack endogenous h-TRβ1. Furthermore, each of these two cell lines has an isogenic counterpart (RKO E6 and MCF-7 E6), which was derived from stable incorporation of the human papillomavirus type 16 E6 gene into genomes (24). E6 gene product stimulates degradation of p53 through a ubiquitin pathway, thereby abrogating p53 functions (18, 25). Fig. 1 confirms that RKO E6 cells had no detectable p53 as compared with RKO stable transfectants with vector only (Fig. 1, lanes 1 and 3). It is known that DNA damage induced by γ-irradiation results in an accumulation of p53 (26). We therefore γ-irradiated RKO cells to indicate that p53 detected in lane 1 was indeed functional. As expected, in γ-irradiated RKO cells, the expression of p53 was increased (Fig. 1, lanes 1 and 2). The protein band with lower molecular weight could be due to a different degree of phosphorylation of p53 (27), which was also observed by others (19, 28). In contrast, no p53 was visible by γ-irradiation of RKO E6 cells (lane 4). This functional impairment is consistent with that reported previously for RKO E6 and MCF-7 E6 (18, 25).

RESULTS

Expression of h-TRβ1 Protein in RKO and MCF-7 Cells Mediated by Recombinant Adh-TRβ1—RKO and MCF-7 cells provided a useful tool to evaluate the functional consequences of the interaction of h-TRβ1 with p53. These two cell lines contain the wild-type p53 but lack endogenous h-TRβ1. Furthermore, each of these two cell lines has an isogenic counterpart (RKO E6 and MCF-7 E6), which was derived from stable incorporation of the human papillomavirus type 16 E6 gene into genomes (24). E6 gene product stimulates degradation of p53 through a ubiquitin pathway, thereby abrogating p53 functions (18, 25). Fig. 1 confirms that RKO E6 cells had no detectable p53 as compared with RKO stable transfectants with vector only (Fig. 1, lanes 1 and 3). It is known that DNA damage induced by γ-irradiation results in an accumulation of p53 (26). We therefore γ-irradiated RKO cells to indicate that p53 detected in lane 1 was indeed functional. As expected, in γ-irradiated RKO cells, the expression of p53 was increased (Fig. 1, lanes 1 and 2). The protein band with lower molecular weight could be due to a different degree of phosphorylation of p53 (27), which was also observed by others (19, 28). In contrast, no p53 was visible by γ-irradiation of RKO E6 cells (lane 4). This functional impairment is consistent with that reported previously for RKO E6 and MCF-7 E6 (18, 25).

Because RKO and MCF-7 cells lack h-TRβ1, recombinant adenovirus containing h-TRβ1 cDNA (20) was used to mediate the expression of h-TRβ1 in these cells. Consistent with that reported by Hayashi et al. (20), we found that more than 90% of...
cells expressed h-TRβ1 as determined by immunofluorescence staining (data not shown). Fig. 2 shows the expression of h-TRβ1 proteins in RKO and MCF-7 cells by Western blotting. Lane 2 shows the expression of h-TRβ1 protein (M, 55,000) in RKO cells infected with Adh-TRβ1, whereas no h-TRβ1 was detected when cells were infected with the control adenovirus lacking the cDNA for h-TRβ1 (Ad-null; lane 2, lane 1). A similar level of expression of h-TRβ1 was also detected in E6-containing RKO cells (lane 4), whereas no h-TRβ1 was seen in Ad-null-infected RKO E6 cells (Fig. 2, lane 3). h-TRβ1 was also expressed in MCF-7 and MCF-7 E6 cells, which were infected similarly with Adh-TRβ1 (lanes 6 and 8, respectively) but not with Ad-null (Fig. 2, lanes 5 and 7). The band with lower molecular weight probably represented the degradation product of h-TRβ1 (23). These results indicated that h-TRβ1 was expressed in these two cell lines via adenovirus infection and that the expression of h-TRβ1 was not affected by the presence of E6 protein.

To confirm that the expressed h-TRβ1 in these cells was functional, we isolated nuclei from cells infected with Adh-TRβ1 or Ad-null and compared their [125I]T3 binding activity. We found that the nuclei isolated from the Ad-null-infected MCF-7, MCF-7 E6, RKO, and RKO E6 cells had no detectable [125I]T3 binding activity, whereas the nuclei isolated from the Adh-TRβ1-infected MCF-7, MCF-7 E6, RKO, and RKO E6 cells bound to [125I]T3 in the range of 67–92 fmol/mg nuclear protein.

**Impairment of the Endogenous p53 Function in MCF-7 and RKO Cells Led to an Increase in T3-dependent Transcriptional Activity of h-TRβ1**—Using exogenous genes transfected into cells, we have previously shown that the transactivation activity of h-TRβ1 was repressed by p53 (8). In the present study, we further investigated whether impairment of p53 functions due to depletion of the endogenous p53 level via E6-enhanced degradation would result in increases in h-TRβ1-mediated transcriptional activity. The isogenic pairs of MCF-7 and RKO cells were transfected with TRE-containing reporters followed by infection with Adh-TRβ1. Bars 2 and 4 of Fig. 3 compare the fold of T3-induced transcriptional activity mediated by p53 TRE in MCF-7 and MCF-7 E6 cells, respectively. It is clear that the transcriptional activity of h-TRβ1 in MCF-7 E6 cells that had reduced p53 was enhanced by ~4-fold. This enhancement was not due to the increased expression of h-TRβ1 in the E6-containing MCF-7 cells, because the expression of h-TRβ1 was identical in MCF-7 and MCF-7 E6 cells (see the expression level of h-TRβ1 in Fig. 2, lanes 6 and 8). These results indicate that lowering the level of p53 relieved the repression effect by p53 on the transcriptional activity of h-TRβ1. A significantly higher transcriptional activity was also detected in RKO E6 cells as compared with RKO cells (Fig. 3, bars 6 and 8). However, the magnitude in the relief of repression was not as high as that seen in MCF-7 cells. This could reflect the fact that a higher level of h-TRβ1 was expressed in MCF-7 cells than was expressed in RKO cells (Fig. 2, lanes 6 and 8 versus 2 and 4). Fig. 3, (bars 1, 3, 5, and 7) indicates the transcriptional activities in the absence of TRs.

**Repression of TR Function by p53 in Growth Hormone-producing GC Cells**—To further establish the functional relevance of the interaction of TRβ1 with p53, we used growth hormone-producing cell lines that were derived from the somatotrophic cells of the anterior pituitary. In growth hormone-producing cells, the synthesis of GH is stimulated by T3 (14, 15), which is mediated by the interaction of TRs with the positive thyroid hormone response elements located in the promoter region of the GH gene (16, 17). Growth hormone-producing cells have long been conveniently used as a model cell line to study the mechanism of thyroid hormone action (14, 15). Using Western blotting, we found that GC cells lacked detectable endogenous p53 in cells treated with or without T3 (Fig. 4, lanes 2–5). Therefore, this cell line provided a tool to examine the effect of p53 on the function of TR by examining the expression of GH mRNA.

We used Ad-p53 to mediate the expression of p53 in GC cells (Fig. 4, lane 6) (19). As shown in lane 7, the expression of p53 was not regulated by T3. For controls, we used GC cells similarly infected, but with the Ad-null in the absence or presence of T3 (lanes 4 and 5, respectively), and no p53 was detected in either condition. The expression of β-actin was also measured, and similar levels of β-actin were seen in uninfected (lanes 2 and 3), Ad-null infected (lanes 4 and 5), and Ad-p53 infected cells (lanes 6 and 7), indicating that under the experimental conditions used, expression of p53 did not affect cell viability.

The T3-induced expression of GH gene in GC cells was determined by mRNA levels using Northern blotting. As shown in Fig. 5, GC cells infected by the control Ad-null virus had the same degree of T3-induced GH mRNA expression as the cells that had not been infected with a virus (~5.5 fold), indicating that the Ad-null virus had no effect on the induction of GH gene.
Cross-talk of TR with p53

FIG. 4. Expression of p53 mediated by infection of GC cells with Ad-p53. GC cells (1.5 × 10⁶/10-cm dish) were not infected (lanes 2 and 3), infected with the control virus Ad-null (lanes 4 and 5), or infected with Ad-p53 (lanes 6 and 7) in the presence (lanes 3, 5, and 7) or absence of T₃ (lanes 2, 4, and 6) as described under "Materials and Methods." Cellular lysates were analyzed by 10% SDS-PAGE, and Western blotting was carried out as described under "Materials and Methods." Lane 1 shows the control p53 protein synthesized by in vitro transcription/translation as a standard marker.

FIG. 5. Effect of p53 on the expression of growth hormone mRNA. GC cells (1.5 × 10⁶/10-cm dish) were not infected, infected with the control Ad-null, or infected with Ad-p53 in the presence or absence of T₃ (100 nM). Total RNA was prepared and Northern blotting was carried out as described under "Materials and Methods." The blots were first probed with 3²P-labeled GH cDNA. Subsequently, the same blot was stripped and reprobed with 3²P-labeled β-actin cDNA. After quantitation of the GH mRNA bands and β-actin mRNA bands, the amounts of GH mRNA were normalized, and the data are expressed as fold of T₃-induced expression of mRNA. The data are the average of three experiments (mean ± S.D.; n = 3). The difference in the fold of T₃-stimulated GH mRNA synthesis is highly significant (**, p < 0.005).

expression by T₃. However, in GC cells that expressed p53, the induction of GH mRNA by T₃ was reduced by ~50%. These results clearly demonstrate that the expression of p53 led to the repression of the stimulatory function of TR on the synthesis of GH mRNA.

DISCUSSION

By two independent systems, the present study demonstrated that p53 cross-talked with TR in the T₃-dependent signaling pathways. In one system, the T₃-dependent transactivation activity of TRs was enhanced by depletion of the endogenous p53 level in both MCF-7 and RKO cells. In another system, the intrinsic function of TRs in T₃-dependent stimulation of the GH gene was repressed by the enhanced expression of p53 in GC cells. These results indicate that p53 is a negative regulator of TR functions. Recently, many TR-interacting proteins have been identified (1–7). Among them, N-CoR (2), SMRT/TRAC (3, 6), and SHP (5) have been shown to be repressors of TRs. The repression action of p53 demonstrated in this study differs in at least three ways from that proposed for N-CoR (2) and SMRT/TRAC (3, 6). First, the repression action of p53 is T₃-dependent, whereas the repression action of N-CoR and SMRT/TRAC only occurs in the absence of T₃. Second, the binding site of p53 is located in the DNA binding domain of TRs (8), whereas the binding site for N-CoR and SMRT/TRAC was shown to be in the D domain (2). Third, the mode of repression by p53 is mediated by inhibiting the binding of TRs to TREs as a result of its binding to the DNA binding domain of TRs (8), whereas the repression action of N-CoR and SMRT/TRAC was proposed to act by locking the TRs in a conformation that is incapable of binding to a co-activator, thereby preventing TRs from functioning as a gene activator (2). Based on these considerations, the repression action of p53 on TRs is most likely independent of that of N-CoR and SMRT/TRAC.

The repression action of p53 on TRs, however, is reminiscent of a recently identified TR-interacting protein, SHP (2). SHP is an orphan nuclear receptor that lacks a DNA binding domain that, in addition to TR, heterodimerizes with several members of the nuclear receptor superfamily. Like p53, SHP prevents the receptors from binding to their hormone response elements and inhibits hormone-dependent transactivation by the receptors with which it interacts (5). SHP was suggested to act as a negative regulator of receptor functions. Even though the repression effect by SHP on endogenous TR target genes has yet to be demonstrated to clearly establish its negatively regulatory role, the findings that TR functions could potentially be negatively regulated by two transcription factors, p53 and SHP, raise an interesting possibility that multiple regulators acting in a similar mode may serve to regulate TR functions in a tissue-specific or development-dependent manner. This notion is supported by the different expression patterns of SHP and p53. SHP is abundantly expressed only in liver, at a much lower level in heart and pancreas, and none in other tissues (5), whereas p53 is expressed in most of the tissues. Together with the different degrees of expression of these two negative regulators at different stages of development, the combinatorial diversities achieved via interaction of TR with p53 and/or SHP could be one of the molecular mechanisms by which TRs mediate the diverse actions of T₃.

The demonstration that p53 could functionally participate in the T₃-dependent signaling pathway has important implications for the understanding of the biology of normal and cancer cells. For example, TRs are known to stimulate cell growth by shortening the duration of the G₁ phase (29–31), whereas p53 has an antiproliferative effect and arrests the progression of cells at the G₁ phase (32). This raises the possibility that p53 could modulate the growth stimulatory effect of T₃. Furthermore, it has been shown that T₃ enhances the x-ray-induced neoplastic transformation in vitro (33). Because mutation of p53 plays a critical role in tumorigenesis, the role of TRs in neoplastic transformation potentially could be modulated by p53. Therefore, our findings have opened a new area in the study of the action of TRs. Furthermore, the identification of p53 as one of the TR-associated proteins further supports the hypothesis that the mediation of the pleiotropic effects of T₃, which requires the cooperation of TRs with a large network of transcription factors and receptors. At present, it is not known whether the function of p53 is also modulated by other members of the receptor superfamily. However, Yu et al. recently found that the transactivation activity of the glucocorticoid receptor detected by a reporter system is also repressed by p53 (34), suggesting that the functions of endogenous p53 could also be modulated by members of the steroid hormone receptors. These findings raise the possibility...
that p53 cross-talks to a large network of nuclear receptor superfamily to regulate cellular functions.

Acknowledgments—We thank Dr. S. Refetoff for the generous gift of Adh-TRβ1, Dr. Zhuangwu Li for help in the preparation of the recombinant adenoviruses, Dr. A. Fornace for RKO/RKO E6 and MCF/MCF-7 E6 cells, Dr. M. Surks for GC cells, and Dr. Xu-Guang Zhu for help with the Northern blot analyses.

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