C-terminal p73 Isoforms Repress Transcriptional Activity of the Human Telomerase Reverse Transcriptase (hTERT) Promoter *

Received for publication, May 10, 2005, and in revised form, October 12, 2005
Published, JBC Papers in Press, October 17, 2005, DOI 10.1074/jbc.C500193200
Tomáš Racek, Nikica Miše, Zhenpeng Li, Anja Stoll, and Brigitte M. Pützer
From the Department of Vectorology and Experimental Gene Therapy, University of Rostock Medical School, Schillingallee 70, 18057 Rostock, Germany

Activation of telomerase is linked to tumorigenesis and has been observed in a variety of human tumors. Previous reports demonstrated that p53 represses human telomerase reverse transcriptase (hTERT), a key component for telomerase activity. The p73 protein displays a tumor suppressor activity similar to p53. In the present study, we examined the effect of transactivation competent p73 isoforms on hTERT expression in p53-negative human H1299 cells. Overexpression of C-terminal p73 isoforms (α, β, γ, δ) resulted in a clear down-regulation of hTERT promoter activity. The strongest inhibitory effect, comparable with p53, was observed for p73β. Moreover, suppression of hTERT expression was also mediated by endogenous p73 after activation of E2F1 in H1299ER-E2F1 cells. Mutations in the Sp1 transcription factor-binding sites of the proximal core promoter region significantly abolished p73-induced repression, suggesting that the effect is mediated by Sp1. Finally, we demonstrate that p73 directly interacts with Sp1, suggesting that formation of a p73-Sp1 complex is the underlying mechanism for p73-triggered inhibition of hTERT expression. Our findings provide additional evidence that p73 mimics p53 in many aspects in cells lacking functional p53, thereby contributing to tumor surveillance.

Telomerase is the enzyme required for the addition of telomeric repeats to the ends of linear chromosomes. It consists of an RNA subunit, a catalytic subunit (hTERT), human telomerase reverse transcriptase) and telomerase-associated proteins. The RNA component of human telomerase provides the template for telomere repeat synthesis (1). Whereas the RNA subunit of telomerase is expressed in most cells, expression of hTERT is observed at high levels in most malignant tumors and cancer cell lines but not in normal tissues (2) and was found to be closely associated with telomerase activity (3). Moreover, ectopic expression of hTERT has been shown to facilitate immortalization of human cells and to be required for the transformation of human primary cells by H-Ras and SV40 large T antigen oncoproteins (4). These findings led to the conclusion that hTERT expression is a rate-limiting step in telomerase activity and a hallmark of cancer. Although telomerase enzymatic activity can be regulated at multiple levels, several studies demonstrated that transcriptional regulation of hTERT is the major mechanism of telomerase regulation. Deletion analyses in reporter assays showed that the 200-bp proximal region of the hTERT promoter (core promoter) is responsible for most of the transcriptional activity (5). Elucidation of the mechanisms governing hTERT expression revealed two general principles for hTERT up-regulation: (i) the activation of the hTERT promoter through oncogenes or viral integration and (ii) the derepression of the hTERT promoter through the loss of tumor suppressors. It has been shown that oncogenes such as c-myc and the human papillomavirus E6 protein activate hTERT transcription (6). Furthermore, abrogation of p53 function by the introduction of mutant p53 induced cellular immortality and telomerase reactivation in mammary epithelial cells (7). In contrast, overexpression of the tumor suppressor gene product p53 was shown to efficiently repress telomerase activity through transcriptional down-regulation of hTERT in a variety of cancer cell lines (8, 9). This effect, which was independent of p53-induced growth arrest and apoptosis (9), requires intact Sp1-binding sites in the core promoter and is attributed to p53-mediated inhibition of Sp1 DNA binding through the formation of a p53-Sp1 complex (8). p53-mediated inhibition of hTERT thus differs from the recently described mechanism of cdc25C promoter repression that involves direct DNA binding of p53 in cooperation with a factor that binds to a GC-box independent of Sp1 (10). However, p53 is the most frequent site of genetic alterations found in human cancers and the absence of functional p53 allows cellular immortalization and predisposes cells to neoplastic transformation (11).

The p53 family member p73 shares substantial structural and functional homology with p53, suggesting a comparable function as a tumor suppressor in human cancer. Unlike p53, p73 gives rise to multiple protein isoforms due to differential mRNA splicing and alternative promoter utilization (12). Among the isoforms generated by alternative splicing at the C terminus are α, β, γ, δ, e, and ω, which share the N-terminal transactivation domain, the DNA-binding domain and the oligomerization domain. Other p73 isoforms resulting from alternative splicing at the N terminus and the use of a second promoter, namely Δα-p73, lack the transcriptional activation domain and act as trans-dominant inhibitors of p53 and p73 (13). When overexpressed, both p73α and p73β have been shown to bind p53 DNA target sites, transactivate p53-responsive genes and induce cell cycle arrest and apoptosis in mammalian cells in a p53-like manner (14). Consistent with many reports indicating that the β isoform is a more potent transcriptional activator than p73α and more effective in inducing apoptosis (15), ectopic expression of p73β was shown to prevent tumor formation in vivo (16). With respect to the similarity between p53 and p73, and the growth-inhibitory, tumor-suppressive function of both molecules, we evaluated the effect of transactivation competent C-terminal p73 isoforms on human telomerase reverse transcriptase promoter activity and examined the mechanism by which p73 regulates hTERT expression.

EXPERIMENTAL PROCEDURES

Cell Lines, Genotoxic Treatment, and Transfections—The H1299 and H1299ER-E2F1 cell lines were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin G/streptomycin sulfate (Invitrogen). Tamoxifen (Sigma, Steinheim, Germany) was used at a final concentration of 1 μM. For genotoxic treatment, cells were exposed to 25 or 50 μM cisplatin (Sigma) or irradiated with 10 or 25 gray. Transfections were performed with Effectene transfection reagent (Qiagen, Hilden, Germany).

Plasmid Construction—For the construction of luciferase (Luc) reporter plasmids containing substitution mutations in Sp1 factor-binding sites 110 bp upstream of the transcription start site of the hTERT gene, site-directed mutagenesis was performed from the pBTDel-408 plasmid using the QuikChange XL kit (Stratagene). The antisense expression plasmid has been described previously (11).

Luciferase Assays—Luciferase activity was measured 36 h post-transfection using a premanufactured luciferase reporter assay system (Promega) and normalized to the total protein concentration in the cell extract.

RT-PCR—Semiquantitative RT-PCR was performed on total RNA prepared by RNeasy Mini Kit (Qiagen). Following DNase I treatment 1 μg RNA was reverse-transcribed using Omniscript RT (Qiagen) and oligo(dT). PCR amplification was performed as described previously (11). A minimum number of cycles was performed to obtain a clear signal within the linear amplification phase. Following specific primer pairs were used: hTERT, 5'-GCAT-CCAGGGCGCAATCTAC-3' (sense) and 5'-TACTGCGCTTGCAGCTC-3' (antisense); p53, 5'-CCAGGGCTCTTCTGTTCTCA-3' (sense) and 5'-GAGCAGAATTCGACACTGATCATCT-3' (antisense); p21, 5'-TTGCACGACGGCCATG-3' (sense) and 5'-AAAGTTCCACCGCTGGG-3' (antisense); GAPDH, 5'-TACACCCTTGTTGGACATGTG-3' (sense) and 5'-CACAGTCCATGTCATTAC-3' (antisense). Primers and Microarray Western Blot Analysis—For immunoprecipitation experiments, 500 μg of lysate from transfected H1299 cells were precipitated using a premanufactured luciferase reporter assay system (Promega) and normalized to the total protein concentration in the cell extract.

This work was supported by Grant 10-1934-Pu3 from the Deutsche Krebshilfe. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed. Tel.: 49-381-494-5068; Fax: 49-381-494-5062; E-mail: bridgette.puetzer@med.uni-rostock.de

2 The abbreviations used are: hTERT, human telomerase reverse transcriptase; Luc, luciferase; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation; 4-OHT, 4-hydroxytamoxifen.

This paper is available online at www.jbc.org. © 2005 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
Chromatin Immunoprecipitation (ChIP) Assay—Chromatin immunoprecipitation assay was performed using ChIP assay kit (Upstate) on H1299 cells transfected with and without p73 expression plasmid. The proteins bound to the hTERT promoter region were co-precipitated with 2 μg of Sp1-antibody (Upstate, Charlottesville, VA) in NET buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 10% glycerol) and subjected to SDS-PAGE. The PCR products co-precipitated with p73 protein were subjected to semi-quantitative RT-PCR analysis on total RNA at 36 h after transfection. The data were normalized to GAPDH values. Co-precipitated p73 isoforms (α, β, γ, and δ) were assayed for luciferase activity. The data shown are averages of triplicates after normalization. Standard deviation is indicated by error bars.

Chromatin Immunoprecipitation (ChIP) Assay—Chromatin immunoprecipitation assay was performed using ChIP assay kit (Upstate) on H1299 cells transfected with and without p73 expression plasmid. The proteins bound to the hTERT promoter region were co-precipitated with 2 μg of Sp1-antibody (Upstate, Charlottesville, VA) in NET buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 10% glycerol) and subjected to SDS-PAGE. The PCR products co-precipitated with p73 protein were subjected to semi-quantitative RT-PCR analysis on total RNA at 36 h after transfection. The data were normalized to GAPDH values. Co-precipitated p73 isoforms (α, β, γ, and δ) were assayed for luciferase activity. The data shown are averages of triplicates after normalization. Standard deviation is indicated by error bars.

Chromatin Immunoprecipitation (ChIP) Assay—Chromatin immunoprecipitation assay was performed using ChIP assay kit (Upstate) on H1299 cells transfected with and without p73 expression plasmid. The proteins bound to the hTERT promoter region were co-precipitated with 2 μg of Sp1-antibody (Upstate, Charlottesville, VA) in NET buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 10% glycerol) and subjected to SDS-PAGE. The PCR products co-precipitated with p73 protein were subjected to semi-quantitative RT-PCR analysis on total RNA at 36 h after transfection. The data were normalized to GAPDH values. Co-precipitated p73 isoforms (α, β, γ, and δ) were assayed for luciferase activity. The data shown are averages of triplicates after normalization. Standard deviation is indicated by error bars.

Stimulation of Endogenous p73 Inhibits hTERT Expression—Next, we examined whether stimulation of endogenous p73 at physiological levels can also repress hTERT promoter activity. We and others (18) have previously shown that p73 is a direct transcriptional target of the cellular transcription factor E2F1. Thus, we generated a H1299 cell line that constitutively expresses E2F1 fused to the modified ligand-binding domain of the murine estrogen receptor, which permits conditional activation of E2F1 and its downstream E2F1 target gene hTERT expression, we transfected H1299ER-E2F1 cells with a p73-antisense expression plasmid construct pBTdel408 containing five Sp1 transcription factor-binding sites and an E-box (17). Transient expression of the p73α and p73β isoforms resulted in a significant repression of the hTERT promoter activity in a dose-dependent manner (Fig. 1A). The shorter p73γ and δ isoforms showed a dose-dependent inhibitory effect. The strongest effect was seen with p73β similar to that observed with wild-type p53. No repression of the hTERT promoter was observed by ΔTA-p73β isoforms (Fig. 1B). Increasing amounts of p73β significantly decreased hTERT mRNA expression at 36 h after transfection as shown by RT-PCR (Fig. 1C) and luciferase reporter assay (Fig. 1D), indicating that hTERT expression is controlled by p73 at the transcriptional level. In contrast, the p53-responsive promoter pGL3-p53 (13) is significantly activated by transactivation-competent p73 isoforms (Fig. 1F).

Results and Discussion

C-terminal p73 Splice Isoforms Repress Transcriptional Activity of hTERT Promoter—We introduced wild-type p73α and the C-terminal splice isoforms p73β, p73γ, and p73δ into p53-null H1299 lung carcinoma cells expressing Sp1 and performed hTERT promoter-luciferase reporter assays using the proximal core promoter construct pBTdel408 (−408 to +5) containing five Sp1 transcription factor-binding sites and an E-box (17). Transient expression of the p73α and p73β isoforms resulted in a significant repression of the hTERT promoter activity in a dose-dependent manner (Fig. 1A). The shorter p73γ and δ isoforms showed a dose-dependent inhibitory effect. The strongest effect was seen with p73β similar to that observed with wild-type p53. No repression of the hTERT promoter was observed by ΔTA-p73β isoforms (Fig. 1B). Increasing amounts of p73β significantly decreased hTERT mRNA expression at 36 h after transfection as shown by RT-PCR (Fig. 1C) and luciferase reporter assay (Fig. 1D), indicating that hTERT expression is controlled by p73 at the transcriptional level. In contrast, the p53-responsive promoter pGL3-p53 (13) is significantly activated by transactivation-competent p73 isoforms (Fig. 1F).
in p73 mRNA expression observed between 36 and 48 h after E2F1 stimulation resulted in increased hTERT transcript levels. Compared with uninduced H1299ER-E2F1 control cells, hTERT reporter activity was significantly inhibited in cells following up-regulation of endogenous p73 (Fig. 2C). In contrast, inactivation of p73 by co-transfection with p73-antisense abolished promoter repression leading to a 35% increase in promoter activity between 36 and 48 h after transfection. These results strongly support that endogenous p73 blocks hTERT activity in p53-negative H1299ER-E2F1 cells.

Identification of hTERT Promoter Elements Responsible for p73-mediated Transcriptional Down-regulation—p73 binds to p53 DNA target sites and transactivates p53-responsive genes in a p53-like manner. However, although we did not assess direct promoter binding of p73, recent findings demonstrated that the lack of p53 binding to the hTERT promoter by Southwestern and chromatin immunoprecipitation experiments (21), thus raising the possibility of an indirect repressive mechanism. To identify the promoter elements responsible for down-regulation, mutants of the reporter plasmid were prepared carrying serial point mutations in the Sp1-binding sites (GC-boxes; Fig. 3A). luciferase assay of H1299ER-E2F1 cells co-transfected with 1 ng of pBTdel408-luciferase reporter and 0.5 ng of p73 expression plasmid in the absence and presence of 4-OHT. For inhibition 2 μg of p73-antisense (p73-AS) expression plasmid was co-transfected. Standard deviation is indicated by error bars.

In p73 mRNA expression observed between 36 and 48 h after E2F1 stimulation resulted in increased hTERT transcript levels. Compared with uninduced H1299ER-E2F1 control cells, hTERT reporter activity was significantly inhibited in cells following up-regulation of endogenous p73 (Fig. 2C). In contrast, inactivation of p73 by co-transfection with p73-antisense abolished promoter repression leading to a 35% increase in promoter activity between 36 and 48 h after transfection. These results strongly support that endogenous p73 blocks hTERT activity in p53-negative H1299ER-E2F1 cells.

Identification of hTERT Promoter Elements Responsible for p73-mediated Transcriptional Down-regulation—p73 binds to p53 DNA target sites and transactivates p53-responsive genes in a p53-like manner. However, although we did not assess direct promoter binding of p73, recent findings demonstrated that the lack of p53 binding to the hTERT promoter by Southwestern and chromatin immunoprecipitation experiments (21), thus raising the possibility of an indirect repressive mechanism. To identify the promoter elements responsible for down-regulation, mutants of the reporter plasmid were prepared carrying serial point mutations in the Sp1-binding sites (GC-boxes; Fig. 3A). luciferase assay of H1299ER-E2F1 cells co-transfected with 1 ng of pBTdel408-luciferase reporter and 0.5 ng of p73 expression plasmid in the absence and presence of 4-OHT. For inhibition 2 μg of p73-antisense (p73-AS) expression plasmid was co-transfected. Standard deviation is indicated by error bars.

p73 Directly Interacts with Sp1—Previous reports demonstrated that p53-mediated transcriptional repression of hTERT requires intact Sp1-binding sites (9) and that this negative regulation is performed through direct protein-protein interaction of p53 and Sp1 (8). To study the mechanism of p73-mediated promoter repression, H1299 cells were co-transfected with the core hTERT promoter luciferase construct together with increasing amounts of Sp1 expression plasmid in the absence or presence of a single concentration of p73 plasmid. As shown in Fig. 4A, the negative effect of p73 closely correlates with the amount of expressed Sp1 in the cells and is significantly reduced at high Sp1 concentrations (~80% decrease with 0.1 μg versus 40% with 2 μg of Sp1 plasmid). Sp1 and p73 protein expression was verified by Western blotting. Next, we examined whether p73 can physically interact with Sp1. In H1299 cells co-transfected with Sp1 and p73B, p73B co-precipitated with Sp1, which was previously shown to interact with full-length p53 protein (Fig. 4B), suggesting in vivo formation of a p73-Sp1 complex. Finally, we determined the effect of p73 on the in vivo binding of Sp1 to the endogenous hTERT promoter in H1299 cells using ChIP analysis. After co-cross-linking with formaldehyde, endogenous Sp1-DNA complexes in H1299 cells were immunoprecipitated with anti-Sp1 antibody and analyzed by PCR using primers specific for the hTERT core promoter. As shown in Fig. 4C, binding of the Sp1 transcription factor to the endogenous hTERT promoter DNA was significantly abolished by the expression of p73 (50.36%; ± 3.1). DNA from the cross-linked extracts pulled down with the Sepharose beads was used as a negative control. No
interaction was detectable between hTERT promoter and HDAC-1 (Fig. 4C, upper panel) as well as between Sp1 and the coding region of actin, not containing Sp1-binding sites (data not shown), which served as negative controls.

Our data strongly support that p73 represses telomerase activity in a p53-like manner through the inhibition of Sp1-mediated transactivation of the hTERT promoter via direct Sp1 binding. However, the β isoform, which has the strongest inhibitory effect on hTERT expression compared with the other C-terminal isoforms, has been shown in many assays to be a more potent transcriptional activator than for example p73α and more effective in inducing apoptosis in tumor cells (22) as well as normal fibroblasts. Since telomerase activity seems to be growth-regulated, an important question is whether the observed direct effect of p73 on the hTERT promoter can be separated from its ability to induce cell cycle arrest and apoptosis. Our results suggest that hTERT down-regulation is not due to the prosopofictive function of p73, as a strong suppression of hTERT mRNA expression was detected in proliferating H1299ER-E2F1 cells within 36 h after E2F1-mediated up-regulation of endogenous p73. During this early time period, we did not observe p73-mediated growth arrest or apoptosis. Thus, our findings suggest a novel function of p73. Regarding the biological significance of this activity, p73-mediated control of telomerase activity might constitute a p53-independent antimitogenic safe-guard mechanism, protecting normal cells against the action of oncogenes.

Acknowledgments—We thank F. Bödicker for generating the hTERT promoter constructs. We also thank C. Afshari and J. C. Barrett for providing the pBTdel408 and B. Vogelstein and G. Melino for providing the p53 and p73 expression plasmids, respectively.

REFERENCES
1. Feng, J., Funk, W. D., Wang, S. S., Weinrich, S. L., Avilion, A. A., Chiu, C. P., Adams, R. R., Chag, E., Allsopp, R. C., Yu, J., Lu, S., West, M. D., Harley, C. B., Andrew, W. H., Greider, C. W., and Villepont, B. (1995) Science 269, 1236–1241.
2. Kim, N. W., Piatiszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Covello, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. W. (1994) Science 266, 2011–2015.
3. Kyo, S., Kanaya, T., Takakura, M., Tanaka, M., and Inoue, M. (1999) Int. J. Cancer 80, 60–63.
4. Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijersbergen, R. L., Brooks, M. W., and Weinberg, R. A. (1999) Nature 400, 464–468.
5. Takakura, M., Kyo, S., Kanaya, T., Hirano, H., Takeda, J., Yutsudo, M., and Inoue, M. (1999) Cancer Res. 59, 551–557.
6. Veldman, T., Liu, X., Yuan, H., and Schlegel, R. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 8211–8216.
7. Goilahon, L. S., and Shay, J. W. (1996) Oncogene 12, 715–725.
8. Xu, D., Wang, Q., Gruber, A., Bjorkholm, M., Chen, Z., Zaid, A., Selivanova, G., Petersen, C., Wilman, K. G., and Pisu, P. (2000) Oncogene 19, 7123–7133.
9. Kanaya, T., Kyo, S., Hamada, K., Takakura, M., Kitagawa, Y., Harada, H., and Inoue, M. (2000) Clin. Cancer Res. 6, 1239–1247.
10. St Clair, S., Giono, L., Varmeh-Ziaie, S., Resnick-Silverman, L., Liu, W. J., Padi, A., Dastidar, J., DaCosta, A., Mattia, M., and Manfredi, J. J. (2004) Mol. Cell 16, 725–736.
11. Ryan, K. M., Phillips, A. C., and Vousden, K. H. (2003) Curr. Opin. Cell Biol. 15, 332–337.
12. Stiewe, T., and Putzer, B. M. (2002) Cell Death Differ. 9, 237–245.
13. Stiewe, T., Theseling, C. C., and Putzer, B. M. (2002) J. Biol. Chem. 277, 14177–14185.
14. Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J. C., Valent, A., Minyi, A., Chaillon, P., Lelias, J. M., Dumont, X., Ferrara, P., McKeon, F., and Caput, D. (1997) Cell 90, 809–819.
15. Sasaki, Y., Morimoto, I., Ishida, S., Yamashita, T., Imai, K., and Tokino, T. (2001) Gene Ther. 8, 1401–1408.
16. Bödicker, F., and Putzer, B. M. (2003) Cancer Res. 63, 2737–2741.
17. Horikawa, I., Cable, P. L., Afshari, C., and Barrett, J. C. (1999) Cancer Res. 59, 826–830.
18. Stiewe, T., and Putzer, B. M. (2000) Nat. Genet. 26, 464–469.
19. Stanelle, J., Stiewe, T., Theseling, C. C., Peter, M., and Putzer, B. M. (2002) Nucleic Acids Res. 30, 1859–1867.
20. Stanelle, J., Tu-Rapp, H., and Putzer, B. M. (2005) Cell Death Differ. 12, 347–357.
21. Shats, I., Milyavsky, M., Tang, X., Stamblowski, P., Erez, N., Brosh, R., Kogan, I., Braunstein, I., Tsukerman, M., Ginsberg, D., and Rotter, V. (2004) J. Biol. Chem. 279, 50876–50885.
22. Zhu, J., Jiang, J., Zhou, W., and Chen, X. (1998) Cancer Res. 58, 5061–5065.