Communication

Protein Phosphatase 2A Inhibits Nuclear Telomerase Activity in Human Breast Cancer Cells*

(Received for publication, April 18, 1997)

He Li, Lin-Lin Zhao, John W. Funder, and Jun-Ping Liu†

From the Baker Medical Research Institute, Commercial Road, Prahran, Victoria 3181, Australia

Most cancer cells have increased levels of telomerase activity implicated in cell immortalization. Activation of telomerase, a ribonucleoprotein complex, catalyzes the elongation of the ends of mammalian chromosomal DNA (telomeres), the length of which regulates cell proliferation. Currently, how telomerase is regulated in cancer is not yet established. The present study shows that telomerase activity is regulated by protein phosphorylation in human breast cancer cells. Incubation of cell nuclear telomerase extracts with protein phosphatase 2A (PP2A) abolished the telomerase activity; in contrast, cytoplasmic telomerase activity was unaffected, and protein phosphatases 1 and 2B were ineffective. Inhibition of telomerase activity by PP2A was both concentration- and time-dependent and was prevented by the protein phosphatase inhibitor okadaic acid. In addition, nuclear telomerase inhibited by PP2A was reactivated by endogenous protein kinase(s) in the presence of ATP, but not in the presence of ATPγS. Furthermore, telomerase activity in cultured human breast cancer PMC42 cells was stimulated by okadaic acid, consistent with a role for PP2A in the regulation of telomerase activity in intact cells. These findings suggest that protein phosphorylation reversibly regulates the function of telomerase and that PP2A is a telomerase inhibitory factor in the nucleus of human breast cancer cells.

One of the critical events in the oncogenic progression of normal human cells is the escape from the limitations on proliferation imposed by cellular senescence. One of the primary mechanisms underlying cellular senescence in normal diploid cells involves controlled telomere shortening in cell division (for reviews, see Refs. 1–4). Telomeres are defined as the ends of all eukaryotic chromosomes comprising an array of tandem repeats of the hexanucleotide 5’-TTAGGG-3’ and its binding proteins. These nucleoprotein structures function to protect chromosomes against exonucleases and ligases, to prevent the activation of DNA-damage checkpoints, and to counter the loss of terminal DNA segments that occurs when linear DNA is replicated. Mutation of telomere repeat, however, blocks chromosome separation suggesting a regulatory role for telomeres in anaphase mitosis (5). Since conventional DNA polymerases cannot replicate the very 5’ end of linear DNA, telomeres shorten as a function of each cell division in normal human somatic cells, contributing to the mechanisms underlying cell senescence (6–9). However, several specialized proteins are involved in the regulation of telomere metabolism, including the yeast double strand telomere-binding protein Rap1 (10) and single strand telomere-binding proteins Rlr8p (11), Cdc13 (12), and Est1 (13), the human duplex telomere-binding protein TRF (14), telomerase, and telomerase-associated protein TP1 (15).

Telomerase, a primary determinant of telomere length, is a ribonucleoprotein complex specific for telomere DNA synthesis (1–4). It is composed of two protein subunits of 95 kDa and 80 kDa in the ciliate Tetrhyymena thermophila (16) and of 120 kDa and 43 kDa in the ciliate Euplotes aediculatus (17). While the 95-kDa subunit binds substrate oligonucleotides, the 80-kDa subunit binds to a telomerase RNA component of 159 nucleotides (16, 18–21). Thus, telomerase uses a portion of this internal RNA moiety as a template to replicate telomeres. Consistent with the shortening of telomeres with each normal cell division, telomerase activity is below detectable levels in normal somatic cells. In most primary human malignancies, however, telomerase is activated by an as yet unknown mechanism. This suggests not only that telomerase activity may be a marker for malignancy but also crucial for unlimited cell division in tumor proliferation through de novo synthesis of telomeres (2, 3, 8).

Recent studies indicate that telomerase activity increases as normal human T cells enter the cell cycle (22, 23), and increased telomerase activity in human promyelocytic leukemic HL60 cells is repressed by cellular differentiation (24–27). In addition, telomerase activity in human breast cancer cells is regulated in a cell cycle-dependent manner, with increased telomerase activity in the S phase and decreased telomerase activity in the G1/M phase (28). These findings suggest that telomerase activity in cell growth and development is reversibly regulated by a molecular switch. Since vertebrate telomerase is a ribonucleoprotein complex with at least two protein components containing multiple potential phosphorylation sites (16), we tested the hypothesis that protein phosphorylation might play a role in telomerase function. The present study shows that nuclear telomerase activity in breast cancer PMC42 cells is specifically abolished by protein phosphatase 2A (PP2A).1 The effect of PP2A is in turn blocked by okadaic acid and reversed by protein phosphorylation through endogenous protein kinase(s), indicating a reversible regulation of telomerase activity by protein phosphorylation and dephosphorylation.

MATERIALS AND METHODS

Cells and Reagents—Human breast cancer PMC42 cells have been described earlier (29). The cells were maintained in the medium of RPMI 1640 supplemented with fetal calf serum (10%), insulin (0.6 μg/ml), hydrocortisone (1 μg/ml), and galactose (0.1%) at an enclosed humidified atmosphere of 95% O2 and 5% CO2 at 37 °C. Cell nuclei were isolated by centrifugation (3000 × g) after homogenization of the cells with a Teflon/glass homogenizer (10 ml), and the cytosolic and mem-

---

* This work was supported by grants from the Australian Research Council and National Health and Medical Research Council of Australia. 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.
† To whom correspondence and reprint requests should be addressed: Baker Medical Research Institute, P. O. Box 348, Prahran, Victoria 3181, Australia. Tel.: 61-3-9522-4333; Fax: 61-3-9521-1362; E-mail: jun-ping.liu@baker.edu.au.

1 The abbreviations used are: PP2A, protein phosphatase 2A; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; ATPγS, adenosine 5’-O-(thiotriphosphate).

This paper is available on line at http://www.jbc.org
branched fractions were separated further by ultracentrifugation (100,000 × g). In some experiments, cells at 80% confluence were treated with okadaic acid (1 μM, from Calbiochem) as indicated. PP2A (>95% pure) was obtained from Biomol Research Laboratories (Plymouth, PA) or partially purified from rat liver as described previously (30). PP1 and PP2B were from UBI (Lake Placid, NY). Taq DNA polymerase, dNTP, and T4 gene 32 protein were from Boehringer Mannheim. [α-32P]ATP was from Amersham. Gel electrophoresis reagents and equipment were from Bio-Rad.

Telomerase Activity Assay—Telomerase activity in the membrane, cytosolic, and nuclear fractions or extracted from whole cells with buffer containing 2.5% CHAPS was determined in duplicate by a slightly modified TRAP assay as described previously (31). Briefly, 1 μg of protein extracts were incubated at 30 °C for 20 min in a 50-μl reaction buffer containing 10 mM Tris, pH 8.3, 0.1 μg of telomerase substrate (5′-AATCCGTTCGACGAGGT-3′), 50 μM each deoxynucleoside triphosphate, 0.5 μCi of [α-32P]dATP, 1.5 mM MgCl2, 1 mM EGTA, 68 mM KCl, 0.05% Tween 20, 1 μg of T4 gene 32 protein. To each reaction was then added 2 μl of Taq DNA polymerase and 0.1 μl of downstream primer CX (5′-CCCTTAACCTACCTTTACCCTAA-3′), followed by a 31-cycle polymerase chain reaction (94 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s). Twenty μl of the polymerase chain reaction was then analyzed for de novo synthesized [α-32P]-telomeres by electrophoresis on a 10% non-denaturing polyacrylamide gel followed by overnight exposure for autoradiography. For relative quantification, telomerase activity was measured by scintillation counting of [32P]-telomeres resolved on non-denaturing polyacrylamide gel electrophoresis and sliced from the gels with autoradiographs as templates. For experimental controls of telomerase activity assay, treatment of telomerase extracts with 2 μg of RNase A or heating at 90 °C for 10 min was routinely included.

RESULTS AND DISCUSSION

Inappropriate telomerase activity has been found to be associated with a variety of human cancers (8). The mechanisms of regulation of telomerase activity have not been established. The primary aim of these studies was to test whether telomerase activity might be regulated by protein phosphorylation. To this end, we have determined effects of the three major cellular protein phosphatases (1, 2A, and 2B) on the activity of telomerase extracted from human breast cancer PMC42 cells. Subcellular fractionation of the cultured cells was performed for nuclear, membrane particulate, and cytoplasmic fractions. Telomerase activity was then examined after incubation of each fraction with exogenous protein phosphatases 1, 2A, and 2B under dephosphorylation conditions (30). Interestingly, PP2A markedly inhibited telomerase activity in nuclear extracts (Fig. 1A), an effect which was concentration-dependent (ED50 10 μM, from Calbiochem) as indicated. PP2A inhibited telomerase activity in the membrane particulate fraction to a lesser extent than in nuclear and appeared inactive in cytosol (Fig. 1C). Substantial levels of telomerase activity were associated with the nucleus (45%) and membrane particulate fractions (38%), compared with 17% in the cytosol (Fig. 1C) of the cultured PMC42 cells. Telomerase activity in different subcellular locations may thus ultimately be achieved by a balance of complex control mechanisms, including the inhibitory effect by PP2A. The inhibition of nuclear telomerase activity by PP2A is specific, since neither protein phosphatase 1 nor 2B affected telomerase activity (Fig. 1B).

Inhibition of telomerase activity by PP2A may reflect direct dephosphorylation of telomerase protein components or indirect activity. Nuclear, membrane, and cytosolic extracts were incubated with 20 units of PP2A for the times indicated. Membrane and cytosolic fractions were obtained by centrifugation (16,000 × g) of the cell lysates after removal of nuclei in the same buffer as above, and the membrane fraction was then suspended in TRAP assay lysis buffer. Telomerase activity was determined in duplicate by counting newly synthesized [32P]-telomeres as described above. Results are from one of two similar experiments.
Telomerase activity is through an ATP hydrolysis-dependent mechanism and that protein phosphorylation by an as yet unidentified protein kinase(s) is likely to mediate this process after dephosphorylation by PP2A. In support of this concept of protein phosphorylation, telomerase reactivation was inhibited by the protein kinase inhibitor H-7 (Fig. 2B). These data unequivocally suggest that protein phosphorylation and dephosphorylation are involved as a molecular switch in up- and down-regulating telomerase activity in the nucleus of human breast cancer cells.

To determine if the PP2A-mediated dephosphorylation event occurs in intact cells, we treated the human breast cancer PMC42 cells with okadaic acid for different periods of time and found that telomerase activity in these cells was markedly stimulated by okadaic acid (1 μM) (Fig. 3). Okadaic acid stimulation of telomerase activity was seen after 30 min and peaked at 2–3 h (Fig. 3). This effect on telomerase activity is thus consistent with the in vitro findings that telomerase is reversibly regulated by protein phosphorylation and dephosphorylation, through unidentified protein kinase(s) and the protein phosphatase 2A. In addition, it is likely that in the control of telomerase activity, at least one of the telomerase protein components or telomerase regulatory proteins is phosphorylated and that protein phosphorylation is required for its activity of telomerase synthesis in cancer, suggesting a molecular mechanism that may explain the reversible regulation of telomerase activity through cell division cycles (22–28). Furthermore, these findings suggest a link between telomerase proteins and PP2A and a potential target of PP2A in controlling telomerase activity in cancer therapy.

Several lines of evidence from previous studies suggest that PP2A is involved in the oncogenic process. PP2A is present at high levels in the nucleus of non-transformed mammalian fibroblasts (33), and it has been suggested to play a regulatory role during the cell cycle (33, 34). Tyrosine phosphorylation of PP2A catalytic subunit by receptor-linked and non-receptor tyrosine kinases inhibits the activity of PP2A (35, 36). Moreover, PP2A is a direct target of at least three distinct DNA tumor virus families, simian virus 40, polyoma virus (37, 38), and adenovirus (39); in addition, binding of the viral oncogenic protein small-t antigen to PP2A inhibits the activity of PP2A and stimulates cell proliferation (40). It is thus conceivable that...
inhibition of PP2A by oncogenic signals or viral tumor antigens may be involved in telomerase reactivation in certain cancers and that the pharmacological stimulation of PP2A dephosphorylation of telomerase in cancer may be of potential therapeutic significance.

Acknowledgments—We thank Bob Whitehead for supplying the breast cancer cell line, Michael Berndt, Alex Bobik, Noel Fidge, Ban-Hock Toh, and Elizabeth Woodcock for their advice, and Zhi-Yong Yang for technical assistance.

REFERENCES

1. Blackburn, E. H. (1992) Annual Rev. Biochem. 61, 113–129
2. de Lange, T. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2882–2885
3. Harley, C. B., Kim, N. W., Prowse, K. R., Weinrich, S. L., Hirsch, K., West, M. D., Bacchetti, S., Hirs, H. W., Counter, C. M., Greider, C. W., Wright, W. E., and Shay, J. M. (1995) Cold Spring Harbor Symp. Quant. Biol. 59, 307–315
4. Greider, C. W. (1996) Annual Rev. Biochem. 65, 337–365
5. Kirk, K. E., Harmon, B. P., Reichardt, I. K., Sedat, J. W., and Blackburn, E. H. (1997) Science 275, 1478–1481
6. Counter, C. M., Avilion, A. A., LeFreuvre, C. E., Stewart, N. G., Greider, C. W., Harley, C. B., and Bacchetti, S. (1992) EMBO J. 11, 2191–2192
7. Levy, M. Z., Allsopp, R. C., Prowse, K. R., Harley, C. W., and Harley, C. B. (1992) J. Mol. Biol. 225, 951–960
8. Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coriell, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. W. (1994) Science 266, 2011–2015
9. Allsopp, R. C., Chang, E., Kashefi-Aazam, M., Rogaev, E. I., Piatyszek, M. A., Shay, J. W., and Harley, C. B. (1995) Exp. Cell Res. 220, 194–200
10. Shore, D. (1994) Trends Genet. 10, 408–412
11. Konkel, L. M., Enomoto, S., Chamberlain, E. M., McCune-Zierath, P., Iyadurai, S. J., and Berman, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5558–5562
12. Garvik, B., Carson, M., and Hartwell, L. (1995) Mol. Cell. Biol. 15, 6128–6138
13. Virta-Pearlman, V., Morris, D. K., and Lundblad, V. (1996) Genes Dev. 10, 3094–3104
14. Chong, L., van Steensel, B., Broccoli, D., Erdjument-Bromage, H., Hanish, J., Tempst, P., and de Lange, T. (1995) Science 270, 1663–1667
15. Harrington, L., McPhail, T., Mar, V., Zhou, W., Oulton, R., Bass, M. B., Arruda, L., and Robinson, M. D. (1997) Science 275, 973–977
16. Collins, K., Kobayashi, R., and Greider, C. W. (1995) Cell 81, 677–686
17. Lingner, J., and Cech, T. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10712–10717
18. Greider, C. W., and Blackburn, E. H. (1987) Cell 51, 887–988
19. Morin, G. B. (1989) Cell 59, 521–529
20. Feng, J., Funk, W. D., Wang, S. S., Weinrich, S. L., Avilion, A. A., Chiu, C. P., Adams, R. R., Chang, E., Allsopp, R. C., Yu, J., Le, S., West, M. D., Harley, C. B., Andrews, W. H., Greider, C. W., and Villeport, B. (1995) Science 269, 1236–1241
21. Blasco, M. A., Rizen, M., Greider, C. W., and Hanahan, D. (1996) Nat. Genet. 12, 200–204
22. Buchkovich, K. J., and Greider, C. W. (1996) Mol. Biol. Cell 7, 1443–1454
23. Weng, N. P., Levine, B. L., June, C. H., and Hodes, R. J. (1996) J. Exp. Med. 183, 2471–2479
24. Sharma, H. W., Sokoloski, J. A., Perez, J. R., Maltese, J. Y., Sartorelli, A. C., Stein, C. A., Nichols, G., Khaled, Z., Telang, N. T., and Narayan, R. (1996) Proc. Natl. Acad. Sci. U. S. A. 92, 12343–12346
25. Bestul, L. J., Brown, C. B., Mitra, Y., Roberston, L. D., and Robenol, K. T. (1996) Cancer Res. 56, 3796–3802
26. Xu, D., Gruber, A., Petersson, C., and Pia, P. (1996) Leukemia 10, 1354–1357
27. Savovsky, E., Yoshida, K., Oktomo, T., Yamaguchi, Y., Akamatsu, K. I., Yamazaki, T., Yoshida, S., and Tsuji, M. (1996) Biochem. Biophys. Res. Commun. 226, 329–334
28. Zhu, X. L., Kumar, R., Mandal, M., Sharma, N., Sharma, H. W., Dingra, U., Sokoloski, J. A., Heiss, R. S., and Narayan, R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6091–6095
29. Whitehead, R. H., Quirk, S. J., Vitali, A. A. Funder, J. W., and Sutherland, R. L., and Murphy, L. C. (1984) J. Natl. Cancer Inst. 73, 643–648
30. Liu, J.-P., Sim, A. T. R., and Robinson, P. J. (1994) Science 265, 970–973
31. Piatyszek, M. A., Kim, N. W., Weinrich, S. L., Hiyama, K., Hiyama, E., Wright, W. E., and Shay, J. W. (1995) Methods Cell. Sci. 17, 1–16
32. Cohen, P. (1989) Annu. Rev. Biochem. 58, 453–508
33. Turowski, P., Fernandez, A., Favre, B., Lamb, N. J., and Hemings, B. A. (1995) J. Cell Biol. 129, 397–410
34. Sontag, E., Numbakhdi-Craig, V., Bloom, G. S., and Mumbay, M. C. (1995) J. Cell Biol. 128, 1131–1144
35. Chen, J., Martin, B. L., and Brautigan, D. L. (1992) Science 257, 1261–1284
36. Chen, J., Parsons, S., and Brautigan, D. L. (1994) J. Biol. Chem. 269, 7967–7962
37. Pallas, D. C., Shahrif, L. K., Martin, B. L., Jaspar, S., Miller, T. B., Brautigan, D. L., and Roberts, T. M. (1990) Cell 60, 167–176
38. Walter, G., Ruediger, R., Slaughter, C., and Mumby, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2521–2525
39. Kleinberger, T., and Shpak, T. (1993) J. Virol. 67, 7556–7560
40. Sontag, E., Fedorov, S., Kamibayashi, C., Robbins, D., Cobb, M., and Mumbay, M. (1993) Cell 75, 887–897