Induction of Transforming Growth Factor-β Receptor Type II Expression in Estrogen Receptor-positive Breast Cancer Cells through SP1 Activation by 5-Aza-2′-deoxycytidine*

(Received for publication, February 25, 1998, and in revised form, April 12, 1998)

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Previous studies suggest that estrogen receptor-positive (ER*) breast cancer cells acquire resistance to transforming growth factor-β (TGF-β) because of reduced expression levels of TGF-β receptor type II (RII). We now report that treatment of ER* breast cancer cells with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-aza-2′-dC) leads to accumulation of RII transcript and protein in three different cell lines. RII induction restored TGF-β response in MCF-7L breast cancer cells as indicated by the enhanced activity of a TGF-β-responsive promoter-reporter construct (pSSTP-Lux). A transiently transfected RII promoter-reporter element (RII-chloramphenicol acetyltransferase) showed an increase in activity in the 5-aza-2′-dC-treated MCF-7L cells compared with untreated cells, suggesting the activation of a transactivator of RII transcription. Using electrophoretic mobility shift assays, the enhanced binding of proteins from 5-aza-2′-dC-treated MCF-7L nuclear extracts to radiolabeled Sp1 oligonucleotides was demonstrated. An RII promoter-chloramphenicol acetyltransferase construct containing a mutation in the Sp1 site was not expressed in the 5-aza-2′-dC-treated MCF-7L cells, further demonstrating that induction of Sp1 activity by 5-aza-2′-dC in the MCF-7L cells was critical to RII expression. Northern analysis indicated that 5-aza-2′-dC treatment did not affect the Sp1 transcript levels. Western blot analysis revealed an increase of Sp1 protein in the 5-aza-2′-dC-treated MCF-7L cells, but there was no change in the c-Jun levels. Studies after cyclohexamide treatment suggested an increase in the Sp1 protein stability from the 5-aza-2′-dC-treated MCF-7L extracts compared with untreated control extracts. These results indicate that the transcriptional repression of RII in the ER* breast cancer cells is caused by suboptimal activity of Sp1, whereas treatment with 5-aza-2′-dC stabilizes the protein thus increasing steady-state Sp1 levels and thereby leads to enhanced RII transcription and subsequent restoration of TGF-β sensitivity.

Transforming growth factor-β (TGF-β)† belongs to a superfamily that includes activins, inhibins, bone morphogenetic proteins, and müllerian-inhibiting substances (1, 2). TGF-β plays an important role in cellular proliferation, differentiation, and synthesis of extracellular matrix proteins (1, 2). Three major TGF-β-binding proteins have been identified. They are referred to as type I (RI), type II (RII), and type III (RIII). RI and RII are glycoproteins of 53 and 75 kDa, respectively, whereas RIII is a 280–330-kDa proteoglycan (3). RI and RII are serine/threonine kinases and form a hetero-oligomeric complex that is required for the TGF-β-mediated signaling cascade (4–7). RIII lacks a signaling motif, and its role appears to be limited to presenting TGF-β to the signaling receptors (8).

One of the important effects of TGF-β is the inhibition of growth of epithelial cells as well as some cancer cells. Because RI and RII are both required for TGF-β-mediated growth suppression, loss of either receptor may contribute to TGF-β resistance and subsequent malignant progression. TGF-β resistance caused by defects in RII expression has been reported in various cell lines (9–11). Previous work has indicated an association between defective RII expression and malignant progression of several cell types (9, 10, 12–14) including breast carcinoma cells (15). RII replacement in breast and colon carcinoma cells restored TGF-β response and reduced malignant behavior (12, 15). It has also been demonstrated that exogenous RII expression in an RII-defective colon carcinoma cell line reversed malignancy (16). These studies underline the importance of both RI and RII as tumor suppressors. Loss of RII expression was observed in gastric cancer cells as well as a subset of colon cancer cells in association with deletions or gene mutations (10, 17). RII repression caused by decreased binding of nuclear proteins to the positive regulatory elements of the RII promoter has been shown to cause TGF-β resistance of adenovirus E1A-transformed mouse keratinocytes (18).

Breast cancer cell lines that express estrogen receptor (ER*) are refractory to TGF-β effects, whereas estrogen receptor-negative (ER-) cells are often TGF-β-sensitive (19). Loss or undetectable expression of RII has been reported to contribute to TGF-β resistance in ER* breast cancer cells (11, 15). Several different ER* MCF-7 strains have been reported in the literature. Comparison of ER* MCF-7 early (MCF-7E) and MCF-7 late (MCF-7L) passage cells from our laboratory has shown that MCF-7E cells express RII and are TGF-β-responsive, but MCF-7L cells lack RII and are TGF-β-resistant, suggesting possible defects at the transcriptional or post-transcriptional level (20). A transiently transfected RII promoter element exhibited markedly decreased activity in the MCF-7L cells compared with untreated controls.
pared with MCF-7E cells, pointing toward a possible defect in transcription.

Gene inactivation caused by methylation of CpG sites in the vicinity of promoter regions has long been associated with tissue-specific and developmentally regulated genes (21). However, recent studies have cited gene methylation as a mode of inactivation of several genes including some that are involved in cell cycle control (14). DNA methyltransferase inhibitors 5-azacytidine and 5-aza-2′-deoxycytidine (5-aza-2′-dC) are the agents used most frequently to reverse methylation and reconstitute the expression of these genes (22).

To delineate the mechanism of RII repression in the MCF-7L and other ER+ breast cancer cells, we have carried out studies using 5-aza-2′-dC and now provide evidence that RII expression is low or undetectable because of suboptimal activity of Sp1 transcription factor. Treatment with 5-aza-2′-dC leads to increased Sp1 steady-state levels as a result of increased protein stability and, consequently, concomitant induction of RII expression. RII expression resulted in restoration of TGF-β sensitivity. These results shed light on a novel mechanism by which epithelial cells escape negative regulatory effects of TGF-β leading to uncontrolled growth and hence tumor formation and progression.

EXPERIMENTAL PROCEDURES

Cell Culture—All of the breast cancer cell lines used were obtained from American Type Culture Collection (ATCC). The BT20 strain in our laboratory has a constitutively active mutated estrogen receptor, hence we refer to it as ER+. Cells were grown in McCoy’s 5A medium supplemented with 10% fetal bovine serum (Sigma), antibiotics, and vitamins (Life Technologies, Inc.). Cultures were main-
cells, but 5-aza-2'-dC-mediated RII expression in MCF-7L cells restored TGF-β1 promoter activity (Fig. 6A). To examine whether the induction of RII after 5-aza-2'-dC treatment is caused by the activation of a transactivator, this promoter element contains two Sp1 binding sites (at −25 bp and −143 bp relative to the start site) and two positive regulatory elements (PRE1, −219 bp/−172 bp; PRE2, +1 bp/+50 bp relative to the start site). 5-Aza-2'-dC-treated MCF-7L cells showed higher promoter activity than control cells, thus suggesting the activation of nuclear proteins that bind to the RII promoter and enhance its activity (Fig. 6B).

EMSAs—To narrow the identity of the proteins that are
enhancing the RII promoter activity in the 5-aza-2'-dC-treated MCF-7L cells, EMSAs were performed using nuclear extracts and the [32P]-labeled oligonucleotides corresponding to the two Sp1 binding sites as well as the two positive regulatory elements (PRE1 and PRE2), which have been recognized previously (18). Oligonucleotides corresponding to the wild type RII promoter PRE1, PRE2, and the two Sp1 sites as well as a mutated Sp1 oligonucleotide were analyzed for binding to nuclear proteins from the 5-aza-2'-dC-treated MCF-7L cells transfected with TGF-β1 (lane 4), which was used as a positive control. Wild type Sp1 oligonucleotides competed with [32P]-labeled Sp1 oligonucleotides for binding to the protein complexes, whereas the mutant Sp1 oligonucleotide could not, thus indicating the specificity of the shifts. To confirm further that the enhanced protein-DNA complex contains Sp1, supershift assays were carried out by incubating the protein-DNA complexes with 2 μg of Sp1 antibody (described under “Experimental Procedures”). Sp1 antibody recognized the Sp1 in the protein-DNA complexes, resulting in a clear shift of the mobility of the protein bound to the [32P]-labeled Sp1 oligonucleotides (Fig. 9).

Effect of 5-Aza-2'-dC on Sp1 Transcription—To determine if 5-aza-2'-dC-mediated Sp1 activity leads to enhanced expression of other Sp1-dependent promoters, we transiently expressed either the wild type or mutant Sp1 site-mutated IGF-II promoter-CAT constructs (Fig. 10A). 5-Aza-2'-dC-treated MCF-7L cells expressed higher wild type RII promoter activity compared with control untreated MCF-7L cells. The Sp1 mutant RII promoter-CAT construct was not expressed (Fig. 10B).

IGF-II Promoter Activity—To determine if 5-aza-2'-dC-mediated Sp1 activity leads to enhanced expression of other Sp1-dependent promoters, we transiently expressed either the wild type or Sp1 site-mutated IGF-II promoter-CAT constructs (Fig. 11A) in 5-aza-2'-dC-treated or untreated MCF-7L cells. The IGF-II promoter contains a distinct TATA box and two Sp1 sites (30). 5-Aza-2'-dC-treated MCF-7L cells exhibited a significantly higher IGF-II promoter activity compared with untreated control cells. The 5-aza-2'-dC-induced IGF-II promoter activity disappeared but retained the basal promoter activity when the Sp1 sites were mutated (−58 Spm IGF-II-CAT). These data further confirm the activation of Sp1 by 5-aza-2'-dC in the MCF-7L cells (Fig. 11B).
of Sp1 mRNA (Fig. 12), suggesting that demethylation was not affecting Sp1 transcription.

Expression of Sp1 Protein—To determine whether 5-aza-2'-dC treatment stimulates the Sp1 protein expression, Western immunoblot was performed using 4 μg each of nuclear extracts from 5-aza-2'-dC-treated and untreated MCF-7L cells. Western analysis showed two protein species of 95 and 105 kDa. The two species are the result of differential post-translational modification of the Sp1 polypeptide (34, 35). Significant increases of both the species were observed in the nuclear extracts of 5-aza-2'-dC-treated MCF-7L cells, whereas there was no change in the c-Jun levels (Fig. 13). Sp1 protein stability studies after treatment with 10 μg/ml cyclohexamide were performed as described previously for this protein (33) to determine if 5-aza-2'-dC stabilizes Sp1 protein indirectly. Sp1 protein from 5-aza-2'-dC-treated MCF-7L cells showed enhanced stability compared with untreated MCF-7L control cells (Fig. 14), thus indicating that the increased steady-state levels of Sp1 protein and oligonucleotide binding activity were the result of indirect actions of the demethylating agent on Sp1.

**DISCUSSION**

Breast cancer cells that express estrogen receptor (ER+) escape negative growth regulation by TGF-β, leading to malig-
nant behavior. Previous work from our laboratory (15) and Kalkhoven et al. (11) indicated that ER− breast cancer cells acquire resistance to TGF-β because of a lack of or inadequate expression of RII. Replacement of RII in a TGF-β-resistant ER+ MCF-7L cell line restored TGF-β response and reduced tumorigenicity in athymic nude mice (15). Reversal of malignancy of a human colon carcinoma cell line was also reported after RII expression (12). Hence, targeting re-expression of RII may offer potential novel approaches for treatment or chemoprevention of breast cancer. In this study, we have examined the ability of the DNA methyltransferase inhibitor 5-aza-2′-dC to restore endogenous RII expression in the ER− breast cancer cells.

Treatment with 5-aza-2′-dC led to induction of RII expression in all three of the ER− breast cancer cell lines examined (Figs. 3 and 4). Significantly, 5-aza-2′-dC-mediated RII induction resulted in restoration of TGF-β response in the MCF-7L cells (Fig. 5). However, the induction of RII expression after 5-aza-2′-dC treatment was not found to be a result of the direct demethylation of the RII gene. This raised the possibility of the involvement of increased activation of a transactivator as a cause for enhanced RII transcription.

RII repression resulting from decreased binding of nuclear proteins to the enhancer regions (PRE1 and PRE2) of the RII promoter has been reported in adenovirus E1A-transformed and untreated MCF-7L cells showed no differences in the nuclear proteins that bind to these enhancer regions (PRE1 and PRE2). The RII promoter lacks a distinct TATA box, and Sp1 has been reported to play an important role in the initiation of transcription from promoters lacking distinct TATA boxes (36). The human RII promoter contains two Sp1 sites at −25 and −143 bp relative to the start site (29). Enhanced RII promoter activity (Fig. 6) as well as the increased binding of nuclear proteins to the 32P-labeled Sp1 oligonucleotides (Figs. 8B and 9) in the 5-aza-2′-dC-treated MCF-7L cells indicated that increased Sp1 activity was induced by 5-aza-2′-dC. An RII promoter-CAT construct with a mutated Sp1 site was not expressed in the 5-aza-2′-dC-treated MCF-7L cells, further demonstrating the specificity of enhanced Sp1 activity resulting from 5-aza-2′-dC treatment (Fig. 10B). Enhancement of Sp1 levels also led to the increased expression of the Sp1-dependent IGF-II promoter in 5-aza-2′-dC-treated MCF-7L cells (Fig. 11B). Consequently, the results presented in this study indicate that suboptimal activity of Sp1 results in transcriptional repression of RII in the ER− breast cancer cells. This appears to have a role in uncontrolled growth and subsequent malignant progression as evidenced by studies showing that RII replacement reverses malignancy in MCF-7L cells (15).

However, 5-aza-2′-dC studies on MCF-7L cells have raised some interesting questions. A similar drug (5-azacytidine) has been reported to increase Sp1 activity without altering protein expression leading to TGF-α transcription in melanoma cells (37). In our study, 5-aza-2′-dC-treated MCF-7L cells did not show any increase in the Sp1 transcript levels (Fig. 12). Thus, Sp1 induction as a result of the demethylation at the Sp1 gene locus was eliminated. However, 5-aza-2′-dC-treated MCF-7L cells exhibited enhanced Sp1 activity as well as increased Sp1 protein levels (Fig. 13). This may result from the effects of 5-aza-2′-dC at a different gene locus, whose product may be

![FIG. 9. Detection of protein-DNA complexes using Sp1 antibody. To confirm that protein-DNA complexes contained Sp1, supershift assays were carried out by incubating the nuclear extract plus 32P-labeled Sp1 oligonucleotide complexes for 15 min at room temperature with 2 μg of Sp1 antibody. A lower mobility complex resulting from the binding of Sp1 antibody to protein-DNA complexes was observed.](image)

![FIG. 10. Panel A, schematic of wild type/mutated −47 RII promoter-CAT construct. The construct contains one Sp1 binding site. Wild type and mutated sequences are indicated. Panel B, wild type and mutated RII promoter-CAT activity in 5-aza-2′-dC-treated MCF-7L cells. The −47 RII promoter-CAT construct with wild type or mutated Sp1 sites was transiently transfected into control or 5-aza-2′-dC-treated MCF-7L cells as described under “Experimental Procedures.” 48 h after transfection, cells were harvested, normalized for β-galactosidase activity, and CAT assays were performed (see “Experimental Procedures”).](image)
Total RNA was isolated from control and 5-aza-2′-dC-treated MCF-7L cells. Transfection, cells were harvested, normalized for MCF-7L cells as described under "Experimental Procedures." 48 h after transfection, IGF-II promoter-CAT constructs were transiently transfected into the control and 5-aza-2′-dC-treated MCF-7L cells as described under "Experimental Procedures." To analyze Sp1 stability, 5-aza-2′-dC-treated MCF-7L nuclear extracts compared with control extracts (10⁵ cells/ml cyclohexamide, and cells were harvested at the indicated time points to isolate nuclear extracts. Nuclear extracts (10 µg) were resolved on a 7.5% SDS-PAGE, transferred to nitrocellulose membrane, and probed with rabbit anti-human Sp1 polyclonal antibody.

levels of Sp1 contribute to repression of RII expression in the MCF-7L cells. In summary, the results of our present study demonstrated that the tumor suppressor gene RII is repressed in the ER− breast cancer cells because of suboptimal activity of Sp1. 5-Aza-2′-dC treatment indirectly stabilizes and activates Sp1, thus leading to enhanced RII transcription and subsequent restoration of TGF-β response. These findings suggest a novel mechanism by which epithelial cells escape the negative growth regulatory effects of TGF-β leading to malignant behavior.

Acknowledgments—We thank Joan Massague for kindly providing the p3TP-Lux construct.

Fig. 11. Panel A, schematic of IGF-II promoter-CAT constructs. The promoter element contains a TATA box and two Sp1 recognition sequences (shown in bold). Panel B, IGF-II promoter-CAT activity in the 5-aza-2′-dC-treated MCF-7L cells. IGF-II promoter-CAT constructs were transiently transfected into the control and 5-aza-2′-dC-treated MCF-7L cells under 80% electroporation efficiency. Transfection, cells were harvested, normalized for β-galactosidase activity, and CAT assay was performed (see "Experimental Procedures").

Fig. 12. Expression of Sp1 transcript in control and 5-aza-2′-dC-treated MCF-7L cells. Total RNA was isolated from control and 5-aza-2′-dC-treated MCF-7L cells as described under "Experimental Procedures," and 10 µg RNA from each sample was resolved on a formaldehyde/agarose gel. RNA was transferred to nitrocellulose membranes and probed with [32P]dCTP-labeled Sp1 cDNA probe.

5-aza-2′-dC (1 µg/ml) - + SP1

Fig. 13. Western immunoblot analysis of Sp1. Nuclear extracts (4 µg) from the control and 5-aza-2′-dC-treated MCF-7L cells were resolved on a 7.5% SDS-PAGE, transferred to nitrocellulose membranes, and probed with rabbit anti-human Sp1 and c-Jun polyclonal antibodies. Sp1 antibody recognizes two protein species of 95 and 105 kDa, which are the result of differential post-translational modifications (40, 41), but c-Jun antibody recognizes a 39-kDa species.

5-aza-2′-dC (1 µg/ml)  +  SP1  95 kDa  105 kDa

Fig. 14. Sp1 stability in control and 5-aza-2′-dC-treated MCF-7L cells. Control (panel A) and 5-aza-2′-dC-treated MCF-7L cells (panel B) were treated with 10 µg/ml cyclohexamide, and cells were harvested at the indicated time points to isolate nuclear extracts. Nuclear extracts (10 µg) were resolved on a 7.5% SDS-PAGE, transferred to nitrocellulose membrane, and probed with rabbit anti-human Sp1 polyclonal antibody.

REFERENCES
1. Massague, J. (1990) Annu. Rev. Cell Biol. 6, 597–641
2. Roberts, A. B., and Sporn, M. B. (1984) in Peptide Growth Factors and Their Receptors (Sporn, M. B., and Roberts, A. B., eds) pp. 419–472, Springer-Verlag, Heidelberg
3. Yingling, J. M., Wang, X.-F., and Bassing, C. H. (1995) Biochim. Biophys. Acta 1242, 115–136
4. Lin, H. Y., Wang, X.-F., Ng-Eaton, E., Weinberg, R. A., and Lodish, H. F. (1992) Cell 68, 775–785
5. Warna, J. L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X.-F., and Massague, J. (1992) Cell 71, 1003–1014
6. Franzen, P., ten Dijke, P., Ichijo, H., Yamashita, H., Schultz, P., Heldin, C.-H., and Miyazono, K. (1993) Cell 75, 681–692
7. Warna, J. L., Attisano, L., Weiser, R., Ventura, F., and Massague, J. (1994) Nature 370, 341–347
8. Lopez-Casillas, F., Wrana, J. L., and Massague, J. (1993) Cell 73, 1435–1444
9. Inagaki, M., Moustaka, A., Lin, Y.-H., Lodish, H. F., and Carr, B. I. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5359–5363
10. Park, K., Kim, S.-J., Bang, Y. J., Yang, J., Park, J.-G., S. Banerji, K., Liu, Brattain, M. G., and Markowitz, S., manuscript in preparation.
11. Mathis, M., Kinzler, K. W., Lutterbaugh, J., Park, K., Bang, Y. J., Lee, H. Y., Park, J.-G., Lynch, H. T., Roberts, A. B., Vogelstein, B., and Markow-
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15. Sun, L., Wu, G., Willson, J. K. V., Zborowska, E., Yang, J., Rajakarunanayake, I., Wang, J., Gentry, L. E., Wang, X., and Brattain, M. G. (1994) J. Biol. Chem. 269, 26449–26455
16. Wang, J., Han, W., Zborowska, E., Liang, J., Wang, X., Willson, J. K. V., Sun, L., and Brattain, M. G. (1996) J. Biol. Chem. 271, 17366–17371
17. Markowitz, S., Wang, J., Myers, L., Parsons, R., Sun, L., Zborowska, E., Fan, R. S., Lutterbaugh, J., Kinzler, K. W., Vogelstein, B., Brattain, M. G., and Willson, J. K. V. (1996) Science 268, 1336–1338
18. Kim, D. H., Chang, J. H., Lee, H. Y., and Kim, S. J. (1997) J. Biol. Chem. 272, 688–694
19. Arteaga, C. L., Tandon, A. K., Von Hoff, D. D., and Osborne, C. K. (1988) Cancer Res. 48, 3898–3904
20. Brattain, M. G., Ko, Y., Banerji, S. S., Wu, G., and Willson, J. K. V. (1996) J. Mammary Gland Biol. Neoplasia 1, 365–372
21. Doerfler, W. (1983) Annu. Rev. Biochem. 52, 93–124
22. Jones, P. A. (1996) Cancer Res. 56, 2463–2467
23. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5294–5298
24. Wu, S.-P., Sun, L.-Z., Willson, J. K. V., Humphrey, L., Kerbel, R., and Brattain, M. G. (1993) Cell Growth Differ. 4, 115–123
25. Gentry, L. E., Lioubin, M. N., Purchio, A. F., and Marquardt, H. (1988) Mol. Cell. Biol. 8, 4162–4168
26. Ruff, E., and Rizzano, A. (1986) Biochem. Biophys. Res. Commun. 138, 714–719
27. Segarini, P. R., Roberts, A. B., Rosen, D. M., and Seyedin, S. M. (1987) J. Biol. Chem. 262, 14055–14062
28. Datto, M. B., Li, Y., Panus, J. F., Howe, D. J., Xiong, Y., and Wang, X.-F. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5545–5549
29. Bae, H. W., Geiser, A. G., Kim, D. H., Chung, M. T., Burmester, J. K., Sporn, M. B., Roberts, A. B., and Kim, S. J. (1995) J. Biol. Chem. 270, 29460–29468
30. Kim, S.-J., Orwuta, U. S., Lee, Y. I., Botchan, M. R., and Robbins, P. D. (1992) Mol. Cell. Biol. 12, 2455–2463
31. Rosenthal, N. (1987) Methods Enzymol. 152, 704–720
32. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) Mol. Cell. Biol. 2, 1044–1051
33. Mortensen, E. R., Marks, P. A., Shistani, A., and Merchant, J. L. (1997) J. Biol. Chem. 272, 16540–16547
34. Jackson, S. P., and Tjian, R. (1988) Cell 55, 125–133
35. Jackson, S. P., McDonald, J. J., Lee-Miller, and Tjian, R. (1990) Cell 63, 155–165
36. Pugh, B. F., and Tjian, R. (1990) Cell 61, 1187–1197
37. Shin, T. H., Paterson, A. J., Grant, J. H. III, Melush, A. A., and Kudlow, J. E. (1992) Mol. Cell. Biol. 12, 3998–4006
38. Han, I., and Kudlow, J. K. (1997) Mol. Cell. Biol. 17, 2550–2558
39. Roo, M. D., Su, K., Baker, J. R., and Kudlow, J. E. (1997) Mol. Cell. Biol. 17, 6472–6480