Hormonal Regulation of the p53 Tumor Suppressor Protein in T47D Human Breast Carcinoma Cell Line*

(Received for publication, September 1, 1995, and in revised form, October 13, 1995)

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Under normal culturing conditions, the T47D human breast cancer cell line expresses progesterone receptor constitutively and is responsive to estrogen. Because the tumor suppressor protein p53 plays a central role in determining genetic stability and cell proliferation, we have examined the effects of 17β-estradiol, the synthetic progestin R5020, and the antiprogestin RU486 on the levels of this protein in T47D cells. Western blot analysis of cellular extracts, performed with a monoclonal antibody capable of quantitatively supershifting a specific p53 response element complex in a gel mobility shift assay, detected a single immunoreactive band representing p53. When cells were grown for 4–5 days in culture medium containing charcoal-treated fetal calf serum, p53 levels declined to 10% of the level seen in the control (no charcoal treatment) group. Supplementation of culture medium containing charcoal-treated fetal calf serum with 0.1–1 nm 17β-estradiol restored p53 to its normal levels. A 4-day treatment of cells with R5020 or RU486 lowered the p53 levels in cells grown in normal culturing conditions to 15 and 30% of control levels, respectively. R5020 and RU486 treatments also caused down-regulation and/or hyperphosphorylation of the progesterone receptor, which correlated with the down-regulation of p53. These observations indicate that in T47D cells, p53 is up-regulated by estradiol while R5020 down-regulates this protein. Since estradiol is known to promote cell proliferation, the induction of p53 observed in this study leads us to propose that estradiol stimulates p53 to regulate proliferation of T47D cells in culture.

The development, growth, and differentiation of human breast is under the influence of a number of hormones including the sex steroids, estradiol (E₂)³ and progesterone. In cases where the breast tissue transforms into a tumorous entity, it often continues to respond to circulating levels of these hormones provided it expresses receptors for the corresponding hormone. While hormone-insensitive malignancies do not appear to express functional receptors, hormone-sensitive cancers may overexpress progesterone receptor (PR) and estradiol receptor (McGuire, 1978). Treatment of breast cancers with hormones or antihormones may suppress the presence of functional receptors, which via activation or inactivation of receptors mediate regression of cancerous tissues. Although breast cancers may initially respond to endocrine therapy, the tissue can transform into a hormone-insensitive entity. The mechanisms underlying the evolution of hormone-sensitive tumors to hormone-insensitive states are not known (Horwitz, 1994). It is, therefore, crucial to determine whether treatment with hormones and/or antihormones might affect a shift toward a progressively more malignant state of breast cancer.

The biological activity of the tumor suppressor protein p53 is associated with suppression of cell growth. It is now widely recognized that p53 may be the most frequently mutated protein in human cancers (Oren, 1992). Because of the crucial role that p53 plays in tumor suppression, we explored the role of E₂ and progesterin in regulating p53 in the T47D cell line, which is responsive to both of these steroid hormones.

We have observed that in T47D cells, E₂, R5020, and RU486 are able to induce a proliferative state, which is detected as an increase in total cell number when cells are cultured in charcoal-treated serum (Iwasaki et al., 1994). Effects of progestins and antiprogestins on the expression of proto-oncogenes have been explored (Schuchard et al., 1993), but there is a paucity of information regarding the influence of steroid hormones on tumor suppressor function in hormone-responsive breast cancer cells. We present evidence here that in T47D cells, p53 is significantly increased upon E₂ treatment while it is down-regulated by both R5020 and RU486.

EXPERIMENTAL PROCEDURES

Materials—The complementary DNA strands containing the p53 response element were synthesized by Keystone Laboratories. AB52 monoclonal antibody (Estes et al., 1987) was received as a gift from Drs. Dean Edwards and Kathryn Horwitz, Denver, CO. PAb240 antibody was purchased from Boehringer Mannheim. Adenosine 5′-triphosphate, ³²P-labeled tetra-(triethylammonium) salt (3000 Ci/mmol) and R5020 were obtained from DuPont NEN. The steroids (progesterone, estradiol, cortisol, and triamcinolone acetonide), phenylmethylsulfonyl fluoride (PMSF), salmon sperm DNA, insulin, penicillin, streptomycin, amphotericin B solution, and activated charcoal were purchased from Sigma. Leupeptin, pepstatin-A, and chymostatin were from Peninsula Laboratories. Dextran T-70 was from Pharmacia Biotech Inc.

Cell Culture and Treatment with Ligands—T47D cells were obtained from American Type Culture Collection. Cells were routinely cultured as described previously (Kodali et al., 1994). Ligands were prepared by the addition of 10⁻¹⁰ M aliquots of various stock solutions prepared in ethanol (0.1% ethanol vehicle) to 10 ml of serum-containing medium at 37°C. Stock solutions of the ligands were prepared in ethanol to a 1000-fold higher concentration. Aliquots (10⁻¹⁰ M) of ligands were added directly to 10 ml of the culture medium. Cells were plated down for 2 days in medium prior to treatment with ligands. Medium and ligands receptor; AB52, anti-PR monoclonal antibody; DHT, dihydrotestosterone; PAB240, anti-p53 protein; mutant; p53-REC, p53 response element complex; PMSF, phenylmethylsulfonyl fluoride; R5020, 17,21-dimethylpregna-4,9(10)-diene-3,20-dione; RU486, 17β-hydroxy-11β-[4-(dimethylamino)phenyl]-17α-(prop-1-yl)estradiol-4,9-dien-3-one; PAGE, polyacrylamide gel electrophoresis.
were added fresh at 2-day intervals. Treatment of fetal calf serum with charcoal suspension (0.5% charcoal, 0.05% dextran T-70 in 10 mM Tris, 1 mM EDTA) was conducted under sterile conditions following our published procedure (Kodali et al., 1994).

**Extraction of Cells**—The culture medium was removed by aspiration, the cells were washed with 10 ml of ice-cold Hanks’ balanced salt solution, and the cells were scraped into 500 μl of extraction buffer (20 mM Hepes, 50 mM NaF, 10 mM Na3MoO4, 8 mM Na2HPO4 2 mM NaH2PO4, 40 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM leupeptin, 30 μg/ml pepstatin-A, 30 μg/ml chymostatin, 0.3 mM PMSF). Cells were burst by the freeze-thaw method via emersion in liquid nitrogen for 20 s and thawed on ice. This was repeated 3 times. After the final thaw, the cell suspension was brought to a final ionic strength of 0.5 M by addition of 4 M NaCl, 20 mM Hepes, pH 7.4, and incubated at 0 °C for 1 h with gentle resuspension of the pellet. A high speed supernatant of the extract was prepared via centrifugation of the burst cells at 35,000 rpm for 45 min at 4 °C and frozen in liquid nitrogen for storage at −80 °C until further use.

**SDS-PAGE and Western Blot Analysis**—High speed supernatants were denatured and 100 μg (10 wells) or 50 μg (15 wells) of total protein per lane were resolved on an 8% polyacrylamide gel under denaturing conditions. Proteins were transferred to Immobilon polyvinylidene fluoride membrane (Millipore) at 30 V for 14 h or 60 V for 7 h in a Tris-glycine buffer system containing 0.025% SDS and 15% methanol. The membrane was blocked for 1 h in TBS-Tween (0.1%) plus 5% Carnation instant nonfat dry milk, and then incubated in the same solution plus a 1:1000 dilution of horseradish peroxidase-conjugated anti-PR antibody AB52 (Estes et al., 1987) for 3 h. The membrane was then washed for 30 min with three changes of TBS-Tween, reblocked for 30 min with TBS-Tween plus 5% Carnation instant nonfat dry milk, and then incubated in the same solution plus a 1:1000 dilution of horseradish peroxidase-conjugated antiserum. Bandswere quantified by transmittance scanning using Scanmaster 3 (Hawtek, Inc., Hudson, NH) and Bio Image whole band analysis (Millipore Corp.). To ensure correct normalization against total protein levels and full transfer of proteins, the polyvinylidene fluoride membranes were stained with Coomassie Blue.

**Gel Mobility Shift Assay**—An oligonucleotide containing the p53 response element (Halazonetis et al., 1993) containing the consensus-specific DNA binding site for p53 (5′-CCGGGCAATTCGGGCAATGTC-3′) was 32P end-labeled using the T4 polynucleotide kinase according to the protocol from U.S. Biochemical Corp. The mixture was heated at 90 °C for 5 min and annealed to the complementary strand by slow cooling to room temperature (23 °C), ethanol-precipitated, and resuspended in 30 μl of 20 mM Hepes, 1 mM EDTA, 0.15 M NaCl, pH 7.4. Reaction mixtures contained approximately 15 μg of total protein, and 5 μg of T47D cellular extracts, 100,000 cpmp of labeled probe, 25 μg Hepes, 12.5 mM NaF, 2.5 mM Na3MoO4, 2 mM NaH2PO4, 0.5 mM NaH2PO4, 100 mM NaCl, 1 mM EDTA, 12.5% glycerol, 0.25 μg leupeptin, 7.5 μl glycerol, 0.25 μg leupeptin, 7.5 μg/ml pepstatin-A, 7.5 μg/ml chymostatin, 75 μM PMSF (total ionic strength, 145 mM) in a total volume of 20 μl. Salmon sperm DNA and antibodies were added 15 min prior to addition of labeled probe to concentrations as indicated in the legends. The DNA binding reaction was incubated at 0 °C for a total of 2 h prior to electrophoresis (pre-electrophoresed 4 h at 175 V) through a 4% non-denaturing polyacrylamide gel using a Trisborate-EDTA buffer system at 125 V for 3 h with tap water cooling.

**RESULTS**

**Characterization of the Specific DNA Binding Activity and Evidence That Anti-p53 Antibody Reacts with p53 from T47D Cells**—The ability of p53 to bind specific DNA sequences is crucial to its transcriptional modulatory function. Inactivation of p53 function appears to result from point mutations that are deleterious to its ability to bind DNA in a sequence-specific manner (Cho et al., 1994). In order to determine whether T47D cells contained a mutant form of p53 and to confirm that the antibody we used to quantify p53 was in fact reacting with the wild type p53 protein, we analyzed the ability of (a) p53 to form a specific p53-p53 response element complex (p53-REc) and (b) the anti-p53 antibody (Transduction Laboratories) and the PAB240, which recognizes mutant p53, to supershift this complex in a gel mobility shift assay (Fig. 1). Incubation of T47D extracts with a high affinity probe (Halazonetis et al., 1993) produced three retarded bands (Fig. 1, lane 2) and no free probe. Addition of nonspecific DNA to the reaction mixture resulted in the appearance of only one band corresponding to p53-REc (Fig. 1, lane 1). The relative decrease in intensities of nonspecific bands upon the addition of nonspecific DNA demonstrates selective p53 binding to the specific DNA sequences in the probe represented by the band marked p53-REc. This band was eliminated by an excess of unlabeled probe (lane 5). The p53-REc was quantitatively supershifted when anti-p53 antibody was added to the reaction mixture (Fig. 1, lanes 6 and 7). However, when antibody PAB240 was added to the reaction mixture, no such supershift was evident (lanes 8 and 9). These data demonstrate the presence of an immunoreactive p53 species in T47D cells, which also binds specifically to the consensus DNA binding site for p53.

**Regulation of p53 by Charcoal-sensitive Serum Factor(s)**—Serum stimulation has been shown to increase the steady state level of p53 in 3T3 cells (Reich and Levine, 1984). High levels of p53 may represent a common feature of transformed, immortalized, and malignant cells (Bartek et al., 1991; Rehsaus et al., 1990). The growth rate of T47D cells is significantly reduced when they are cultured in medium containing charcoal-treated serum, which apparently becomes depleted of small hydrophobic factors such as steroids (Iwasaki et al., 1994). Consequently, we were interested in determining whether the factor(s) removed by charcoal treatment had an effect on the levels of p53. Cells were plated in whole serum for 2 days and then grown in medium containing charcoal-treated serum for 3–8 days (Fig. 2). All cells were grown for 10 days with the number of days in charcoal-treated serum represented at the top of each lane. The control (C) cells were cultured for the entire period in medium that was not treated with charcoal. Whole cell extracts were prepared and p53 protein levels were determined by Western analysis. Culturing the cells in char-
analyzed for protein concentration, and a total of 100 μg of protein/lane for each condition was subjected to SDS-PAGE and Western blot analysis as described under "Experimental Procedures." The extracts were analyzed for protein concentration, and a total of 100 μg of protein/lane for each condition was subjected to SDS-PAGE and Western blot analysis as described under "Experimental Procedures."

Coal-treated serum for 5 days resulted in minimal levels of p53; this period appeared to be sufficient to deplete the factor(s) that maintained high levels of p53. Culturing the cells in coal-treatment serum for longer periods of time (up to 8 days) did not change the levels of p53. This observation suggests that p53 is expressed at a basal level in T47D cells, which does not depend on factors removed from serum by coal-treatment. This basal level of p53 is represented in lanes marked 5-8. Under these conditions p53 remains constant at approximately 10% of the control value.

 Estradiol Reverses the Effect of Coal-treatment of Serum—When cells were cultured in coal-treatment serum supplemented with R5020, E2, cortisol, or dihydrotestosterone (DHT), a physiological concentration (1 nM) of E2 reversed the effect of coal-treatment on the p53 levels. DHT had a similar effect but at a relatively high concentration (1 μM) (data not shown). This concentration of DHT has been shown to activate the estradiol receptor and stimulate growth of the MCF-7 breast cancer cell line (Zava and McGuire, 1978) and may, therefore, be acting similarly in T47D cells. When the cells were treated with varying concentrations of E2 (1–1000 pm) to determine its lowest effective concentration (Fig. 3), a relatively low concentration of E2 (100 pm) was sufficient to reverse the effect of coal-treatment of serum on the levels of p53. It is widely known that coal-treatment removes free steroids from cellular extracts and serum samples. We believe that the coal-treatment of the serum resulted in the reduced p53 expression due to the removal of E2, since low concentrations of this hormone were sufficient to raise p53 to the level seen in the control samples (Fig. 3, C).

PR Binding Ligands Decrease the Levels of p53 in Cells Grown in Whole Serum—The effects of progestins and anti-progesterone were examined on the levels of p53 in T47D cells grown in whole serum. We had observed that culturing T47D cells for 7 days in coal-treatment serum reduced PR by at least 70% as determined by Western blot analysis. Thus, to observe any effect of PR ligands on the levels of p53, cells were grown in whole serum to maintain the levels of PR. When T47D cells were grown in whole serum medium and were treated with 10 nM R5020, the synthetic glucocorticoid agonist, triamcinolone acetonide, or RU486, the levels of p53 were significantly decreased (Fig. 4, upper panel). The natural hormones progesterone and cortisol had no effect. Activation of PR upon treatment of T47D cells with RU486 or R5020 correlates with hyperphosphorylation of PR, which slightly retards the mobility of PR-A and PR-B during SDS-PAGE (Beck et al., 1992; Takimoto et al., 1992). Treatment of cells with R5020 leads eventually to down-regulation of PR (Wei et al., 1988; Elashry-Stowers et al. 1988). We, therefore, analyzed the phosphorylation state and the levels of PR from the same cellular extracts by Western blot (Fig. 4, lower panel) to determine the effect of the ligands on PR. Decreased levels of p53 (Fig. 4, upper panel) correlated well with down-regulation or phosphorylation of PR (compare p53 levels and down-regulation with the upshifted PR). Conditions where PR was unaffected by ligand also had no effect on the levels of p53. These data indicate R5020 down-regulates p53 whereas estradiol treatment of T47D cells up-regulates the tumor suppressor protein.

**DISCUSSION**

The wild type protein product of the tumor suppressor gene, p53, is a nuclear phosphoprotein, which functions as a transcription factor directly regulating the expression of factors involved in cell cycle control (Kastan et al., 1992) and programmed cell death (Miyashita and Reed, 1995; Miyashita et al., 1994; Oltvai et al., 1993). The ability of p53 to act as a transcription factor is determined by its sequence-specific DNA binding activity (Kern et al., 1991; Hupp et al., 1992; Cho et al., 1994). The occupancy of response elements by p53 and its ability to trans-activate responsive genes would at least partially depend on its intranuclear concentration. Thus, measured levels of this protein are an important determinant of its activity. Since we examined the effects of steroids (E2, R5020, and RU486) on the levels of p53 using Western blot analysis, it was important to demonstrate the specific reactivity of the anti-p53 antibody. For this, we employed the gel mobility shift assay. The observation that a specific p53-RE could be detected in whole cell extracts indicates that at least with respect to its specific DNA binding activity as shown in Fig. 3, p53 in T47D cells is normal. It is not surprising that PAb240, an antibody that recognizes mutant forms of p53, neither blocked the formation nor supershifts the p53-RE. Our results are in agreement with a similar observation reported in the literature.
ture on the inability of PAb240 to supershift the p53-REc (Halazonetis et al., 1993). The anti-p53 antibody appeared to increase the affinity of p53 for its response element as well as supershift the p53-REc (Fig. 1). Antibody-induced increases in the specific DNA binding activity of p53 have been reported previously (Halazonetis et al., 1993, Hupp et al., 1992). The same antibody we used to supershift the p53-REc was also capable of detecting a single protein band in a Western blot analysis of T47D extracts, which migrated at the expected molecular weight.

Treatment of cells with R5020 or RU486 altered the levels of p53 when cells were cultured in charcoal-treated serum, but the effects were not dramatic (data not shown). Charcoal treatment of serum appears to remove, among other components, a hormone or a factor that induces PR. Accordingly, lowering the levels of PR by charcoal treatment of serum limits progesterin effects. We believe that among a number of possible heterogeneous compounds susceptible to removal by charcoal treatment, $E_2$ may be a prime candidate. We have demonstrated in this report that $E_2$ is sufficient to restore the levels of p53 and recently have determined it is also sufficient to restore PR levels. This may explain how PR-mediated down-regulation of p53 might occur with R5020 and RU486 treatment. For optimal detection of the effects of R5020 and RU486 on p53 levels, the cells require priming by $E_2$ to raise the PR and p53 to effective levels. The $E_2$-induced increase in p53 levels in the absence of PR stimulation raises p53 levels as a check on the $E_2$-induced proliferative state of T47D cells. However, when PR is activated by R5020 or RU486 under estrogenized conditions, PR action predominates and p53 levels are decreased. A PR-mediated mechanism for reducing p53 in T47D cells would thus require estrogenic stimuli. However, demonstration of a direct link between PR and p53 levels must await future investigations.

RU486 appears to be acting as a PR agonist in this system. The agonist effects of RU486 could be explained on the basis that the activation function (AF1) in the N-terminal transcriptional activation domain of PR is sufficient to mediate down-regulation of p53 and that the ligand-dependent activation function (AF2) in the hormone binding domain, which would be inhibited by RU486, is not required (Gronemeyer et al., 1992). Alternatively, RU486 has been shown to act as an agonist when cAMP levels are increased in T47D cells (Beck et al., 1993). Because $E_2$ has been shown to increase intracellular cAMP levels in cultured breast cancer cells (Aronica et al., 1994), it is possible that the levels of $E_2$ in the whole serum or upon addition to charcoal-stripped serum were sufficient to convert RU486 to an agonist.

Because triamcinolone acetonide, but not cortisol, down-regulated p53 and caused an upshift and down-regulation of PR, it would appear that the former binds PR and activates it in the T47D cells. The failure of the natural PR ligand progesterone to mimic the effects of the potent progesterin R5020 is probably due to its rapid metabolism in T47D cells whereas R5020 is more stable (Horwitz et al., 1986). The latter possibility is supported by the observation that progesterone treatment neither induces upshift of PR nor its down-regulation (Fig. 4).

Although studies on the effects of steroids on p53 function are limited, recent reports indicate a precedence of steroid hormone regulation of p53. Withdrawal of androgen appears to increase the levels of p53 in the epithelial and stromal cells of the rat ventral prostate resulting in increased levels of apoptotic cells (Banerjee et al., 1995). Increased levels of p53 in this case might be due to inactivation of androgen receptor as a result of DHT withdrawal. This effect may be analogous to our observations of PR-mediated alterations in p53 levels in T47D cells and is consistent with the results that show PR binding ligands specifically reduce the p53 level in T47D cells. Whether androgen, estrogen, or progesterone receptors directly regulate gene expression related to p53 transcription or whether the mechanism is indirect or post-transcriptional remains to be determined.

Acknowledgments—We thankfully acknowledge the gifts of antibody AB52 received from Drs. Dean Edwards and Kathryn Horwitz. RU486 was generously provided by Roussel Uclaf. The secretarial assistance of Rita Perris is greatly appreciated.

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