Identification of an Estrogen-inducible Phosphatase (PP5) That Converts MCF-7 Human Breast Carcinoma Cells into an Estrogen-independent Phenotype when Expressed Constitutively*

Received for publication, April 19, 2001
Published, JBC Papers in Press, April 30, 2001, DOI 10.1074/jbc.M103312200

Gudrun Urban‡, Teresa Golden‡, Ileana V. Aragon‡, Jonathan G. Scammell§, Nicholas M. Dean‡, and Richard E. Honkanen‡‡

From the Departments of ‡Biochemistry and Molecular Biology and §Pharmacology and Comparative Medicine, University of South Alabama, Mobile, Alabama 36688 and ¶ISIS Pharmaceuticals, Carlsbad, California 92008

The proliferation of many estrogen receptor (ER)-positive breast cancer cells depends on estradiol, and tumors arising from these cells are often responsive initially to treatment with selective ER modulators, which produce an antiestrogen effect. However, tumors that are refractory to the antiestrogenic effects of selective ER modulators often reemerge, and the prognosis for these patients is poor because of the lack of additional effective therapy. Accordingly, deciphering the cellular events associated with estrogen-dependent growth and the subsequent outgrowth of tumors with an estrogen-independent phenotype is of considerable interest. Here we show that the expression of PP5, an evolutionarily conserved Ser/Thr phosphatase that functions as an inhibitor of glucocorticoid- and p53-induced signaling cascades leading to growth suppression, is responsive to 17β-estradiol (E2) in ER-positive human breast carcinoma cells (MCF-7). Northern analysis revealed that E2-induced PP5 expression is blocked by treatment with tamoxifen, and a consensus ER recognition element was identified in the PP5 promoter. The PP5-ER recognition element associates with human ERs and confers E2-induced transcriptional activation to reporter plasmids. The specific inhibition of PP5 expression ablates E2-mediated proliferation in MCF-7 cells without having an apparent effect on E2-induced expression of c-myc or cyclin D1. Thus, although critical for cell growth, PP5 likely acts either downstream or independently of c-Myc and Cyclin D1. To further characterize the role of PP5 in E2-regulated growth control, we constructed stable MCF-7 cell lines in which the expression of PP5 was placed under the control of tetracycline-regulated transactivator and operator plasmids. Studies with these cells revealed that the constitutive overexpression of PP5 affords E2-dependent MCF-7 cells with the ability to proliferate in E2-depleted media. Together, these studies indicate that E2-induced PP5 expression functions to enhance E2-initiated signaling cascades leading to cell division and that aberrant PP5 expression may contribute to the development of MCF-7 cells with an estrogen-independent phenotype.

Breast cancer is a leading cause of cancer mortality among women with an annual rate as high as 1.04/1,000 women in the United States (1). Approximately 50% of all breast cancer cells express receptors that recognize estrogen, and in these estrogen receptor (ER1)-positive cells 17β-estradiol (E2) has a potent mitogenic effect (2). The growth-promoting effects of E2 influence both the initiation and progression of cell growth, stimulating resting noncycling (G0 phase) cells to enter the cell cycle and accelerating G1-S phase progression (3–6). Proliferation is initiated by the activation of ERs, which act as hormone-regulated transcriptional factors that control the expression of estrogen-responsive genes (7–8). Several ER-responsive genes (e.g. c-myc and cyclin D1) have been linked to the propagation of a proliferative response, and agents that bind ERs and modify estrogen actions (such as SERMs) effectively inhibit E2-induced gene expression in many ER-positive tumors. Accordingly, SERMs have emerged as important drugs for the clinical management of hormone-responsive breast cancer (9). Nonetheless, eventually most breast tumors that initially respond to SERM therapy develop resistant metastases, and the prognosis for these patients is poor because of the lack of additional effective therapy (10).

The mechanism(s) underlying acquired SERM resistance is poorly understood, controversial, and likely multifactorial. Modification of ER structure/function (11–13), altered pharmacology of the SERMs (14–15), and paracrine interactions of ER-induced growth factors (16) have all been proposed to contribute to the development of refractory tumors. In addition, hormone-independent mechanisms can activate ERs. Ligand-independent ER activation has been observed after treatment with dopamine, polypeptide growth factors that activate mitogen-activated protein kinase pathways, cell cycle regulatory proteins, and okadaic acid, a potent semi-selective Ser/Thr protein phosphatase inhibitor (17–19). Together, these findings suggest that the regulation of ER function is controlled by both ligands and “cross-talk” from signaling cascades initiated by the activation of growth factor receptors.

Because reversible phosphorylation has been implicated in the regulation of both ligand-dependent and -independent ER-mediated growth control (17–19), it seems likely that both protein kinases and protein phosphatases influence the growth of...
hormone-responsive tumors. To characterize the roles of Ser/Thr protein phosphatases, inhibitors such as okadaic acid, cantharidin, and fostriecin have emerged as powerful tools. These compounds have potent inhibitory activity against the type 1 (PP1) and 2A (PP2A) family of phosphatases, and studies with okadaic acid indicate that sensitive PPases influence both receptor activation and cross-talk with cascades induced by the activation of growth factor receptors (17–19). However, in humans there are four pharmacologically indistinguishable isoforms of PP1 (PP1α, PP1β, PP1γ, and PP1δ) (20, 21) nearly identical isoforms of PP2A (PP2Aα and PP2Aβ) (22, 23), and three structurally related phosphatases (PP4 (24), PP5 (25), and PP6 (26)) that are all sensitive to okadaic acid, cantharidin, and fostriecin. Therefore, it is difficult to distinguish the actions of individual PPases using the currently available inhibitors.

Studying the cellular roles of PP5 has proven particularly difficult, in part because no physiological substrates for PP5 have been identified. In addition, in crude cell homogenates PP5 resides predominately in an inactive state (27), which represents <1% of the measurable PPase activity. This makes changes in the activity of PP5 impossible to detect in the high background of PP1/PP2A/PP4. To illuminate the cellular roles of human PP5, we have developed chimeric antisense 2'-O-(2-methoxy)ethylphosphothioate oligonucleotides that are capable of specifically inhibiting the expression of PP5 via RNAse H-mediated degradation of PP5 mRNA (28). The lead compound targeting PP5, ISIS 15534, inhibits the expression of human PP5 at nanomolar concentrations (28, 29). More importantly, ISIS 15534 has no apparent effect on the structurally related PPases (28), which allows the use of ISIS 15534 to study how the specific suppression of PP5 expression influences the biological activity of cultured human cells.

Previous studies have shown that ISIS 15534 produces a growth-inhibitory effect in some but not all human tumor cells (28). More recent studies have revealed that the suppression of PP5 expression results in enhanced glucocorticoid receptor (GR) activity, the hyperphosphorylation of p53, and a marked increase in the expression of the p21-cyclin-dependent kinase inhibitor protein (29). This suggests that PP5 acts as suppressor of GR- and p53-induced growth-suppressing signaling cascades. Here we show that the expression of PP5 is responsive to estrogen in human ER-positive human breast carcinoma cells (MCF-7). The suppression of PP5 expression inhibits cell growth without having an apparent effect on the expression of c-myc or cyclin D1. In contrast, the constitutive overexpression of PP5 converts estrogen-dependent MCF-7 cells into an estrogen-independent phenotype. Together, these studies suggest that PP5 acts to link ER- and GR-dependent growth control signaling networks, with estrogen-induced PP5 expression acting to aid the growth-promoting effects of estrogen by suppressing GR-induced signaling cascades, leading to growth arrest.

EXPERIMENTAL PROCEDURES

Materials—17 β-estradiol, tamoxifen, propioudion, 5,6-tetrapyrene, and 5418 were purchased from Sigma. The Trizol reagent was purchased from Life Technologies, Inc.

Cell Lines and Cell Culture—MCF-7 cells were obtained from the American Type Tissue Collection. The cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 0.1 μg/ml streptomycin, and 25 units/ml penicillin. Unless indicated otherwise, all cell lines were passaged when 90–95% confluent. In estrogen depletion studies, the cells were cultured in phenol red-free DMEM supplemented with 10% serum obtained from gelded horses (GHS). Clinical chemical analysis revealed that the GHS used in these studies contained 9.6 mg/ml 17 β-estradiol.

Analysis of Cell Growth and FACS Analysis—Cell numbers were determined with a cell counter (Coulter Counter ZM) after detachment from the dish by a brief treatment with trypsin. For cellular staining with propidion, cell cultures were detached gently with trypsin, collected by centrifugation, and resuspended in 0.5 ml of PBS containing 50 μg/ml propidion iodine and 0.5 ml of Vindelov reagent (0.01 M Tris, 10 mM NaCl, 75 μM propioudion, 1 mM/μl Igepal and 700 units/μl RNAse adjusted to pH 8). The propioudion iodine-stained cells were kept in the dark at 4 °C for 30 min and then with 582/520 filter in the front to the FL2 detector. Multivariate data acquisition and analysis were performed with CellQuest software (Becton Dickinson Immunocytometry Systems).

Antisense Oligonucleotide Synthesis and Assay for Oligonucleotide Inhibition of PP5 Expression—Phosphorothioate deoxoygulonucleotides and 2'-O-(2-methoxy)ethylphosphothioate deoxoygulonucleotides were synthesized and purified as described previously (30, 31). Antisense oligonucleotide-mediated inhibition of PP5 expression and Northern blot analysis were performed as described previously (28–31). Briefly, cells that reached ~60–70% confluency in 60-mm dishes were washed once with DMEM and then treated with a solution (1 ml) of DMEM containing oligonucleotides at the indicated concentrations and 15 μg/ml of 17β-estradiol added for 18 h. In some experiments, the cells were kept for 4 h in the presence of 10% FCS or GHS. After 18 h, the cells were scraped in Trizol reagent (Life Technologies, Inc.), and the total RNA was isolated. For Northern blot analysis, 15 μg of RNA was separated on a 1% agarose gel containing formaldehyde and transferrred to a Duralon-UV (Stratagene) membrane. Hybridization was conducted in the presence of 50% formamide at 42 °C. 32P-Labeled cDNA probes were generated by random labeling with Klenow enzyme (Decaprime kit, Ambion). The membranes were subjected to two short stringency washes with 2× SSC at room temperature after two high stringency washes with 0.1× SSC at 55 °C. Hybridization was visualized by autoradiography. For quantification of hybridization signals, autoradiograms were scanned, and the scanned signals were integrated using Scion Image software (3b beta release, Scion Corporation). The signals were normalized to GAPDH to account for any inconsistencies in loading.

RACE Analysis of PP5 Transcripts—RACE was performed using the Advantage 2 Taq PCR system (CLONTECH) with 4% dimethyl sulfoxide added to the reaction mixtures. The first round of PCR was conducted using the AP1 primer supplied in the kit and RH261 (5′-GCTCAGCACACTCAGTCCTCTCGCCCTCCGC-3), a PP5-specific reverse primer present in exon 1 of human PP5. For the first round of PCR, 0.5 μg of cDNA was used as a template, and the PCR reaction was conducted as follows: 94 °C for 1 min; five cycles of 94 °C for 20 s and 70 °C for 3 min; five cycles of 94 °C for 20 s and 70 °C for 3 min; 30 cycles of 94 °C for 30 s and 68 °C for 30 s; 72 °C for 2 min; 5 μl of a 1.50 dilution from the primary PCR was then used as template for the second round nested PCR-RACE. The reaction conditions were the same as described for the first round except the final cycling set, in which only 25 cycles were performed. For the second round of PCR, the AP2 primer supplied in the kit was used in combination with RH252 (5′-CCAGCTTCAAAAGCAGCCTACCTGCAGCAGC-3), a PP5-specific reverse primer starting four base pairs upstream of the human PP5 coding region and containing a 5′-HindIII site to facilitate cloning. The resulting products from the second round were separated by agarose gel electrophoresis and transferred to a Duralon UV membrane for Southern analysis. A 560-base pair region upstream (~640 to ~81) of the PP5 coding region was used as a probe. A strongly hybridizing band was identified, and the corresponding band (visualized by ethidium bromide staining) was isolated and purified from the gel. The 750-base pair band was then digested with NotI (contained in the AP2 primer) and HindIII (contained in the RH252 primer) and cloned into Bluescript SK(+) (Stratagene). The sequencing analysis was performed using T3 and T7 primers.

Construction of PP5- Luciferase Reporter Plasmids—To obtain the 5′-flanking region of human PP5, sense oligonucleotides corresponding to the indicated 5′-flanking regions of human PP5 (Fig. 2, ~758, ~843, ~184, and ~110) were employed in combination with an antisense oligonucleotide (RH252, described above) in PCR of human genomic DNA. The sense primers employed were as follows: RH250 (~110) 5′-CCGGATCCTGAAGGACCTGACCTGCTGCGA-3′; RH254 (~184) 5′-CCGGATCTCGTCACATTTCGCTCCGCGCCGTCCTG-
PP5 Converts MCF-7 Cells into an Estrogen-independent Phenotype

RESULTS

Estrogen Depletion Is Associated with G1 Growth Arrest and a Decrease in PP5 mRNA Levels—MCF-7 cell growth depends on estrogen, and when placed in serum-free medium or medium supplemented with serum depleted of estrogen by charcoal stripping or the use of serum-free medium, the growth rate of MCF-7 cells is greatly suppressed. The second system employed a dual-luciferase reporter assay system using the constructs described above. After 24 h, the medium was replaced with estrogen-depleted medium (see above), in which the cells were maintained for 48–60 h as indicated. On the third day, the cells were transfected with the indicated plasmids using the LipofectAMINE reagent (Life Technologies, Inc.) as described previously (29). The transfection mixture was removed 4 h later, and the cells were returned to estrogen-free medium overnight. The next morning, 17β-estradiol (10 nM) was then added to the experimental plates, and the cells were incubated for another 24 h. Two assay systems were employed to confirm the results, and the amount of DNA transfected depended on the assay system used. In the first system, the enhanced luciferase assay kit (PharMingen) was used; 1.5 µg of each vector was employed and the luciferase activity was measured using a Monolight 2010 Luminoimeter. The ratio of activity to total protein (as measured with a Bio-Rad DC assay) was then calculated. The second system employed a dual-luciferase reporter assay system using these studies. 1.5 µg of the experimental vector and 0.5 µg of pRL-cytomegalovirus Renilla (Promega) were co-transfected into the cells. Luciferase and Renilla activity were then measured in a Turner Designs luminoimeter. The ratio of luciferase to Renilla activity was calculated and plotted. All statistical calculations were performed using Microsoft Excel.

Cloning of Stable Cell Lines Overexpressing PP5—Western analysis was performed as described previously (28). Double-stable cell lines overexpressing PP5 were developed by the stable transfection of MCF-7 Tet-off cells (CLONTECH) with a pBluescript growth factor-derived plasmid (CLONTECH) encoding the full-length human PP5. Clones were grown in the presence of 2 µg/ml tetracycline (to suppress PP5 expression) and selected for by resistance to G418. Surviving cell lines were then screened by both Northern and Western analysis for the presence of the estrogen-responsive PP5 mRNA and protein expression, respectively. Several clones from different plates were obtained. Three cell lines (MCF-7-PP5 h1, MCF-7-PP5k1, and MCF-7-PP5l1), in which the level of PP5 mRNA expressed upon the removal of tetracycline was similar to that produced by the addition of 1–10 nM 17β-estradiol in the parental MCF-7 cells, were chosen for further study.

Isolation and Characterization of the Human PP5 5′-Flanking Region—Sequence analysis of a 1.9-megabase region of human chromosome 19q31.2 revealed an ~43.5-kilobase region containing 13 exons that encode PP5 and an ~92-kilobase region 5′ of exon 1 (BAC 82621, GenBank™ accession number AC007193). 5′-RACE analysis of human cDNA performed using PP5-specific PCR primers contained in exon 1 identified four clones containing the 5′ region of the human PP5 gene. Sequence analysis revealed that two clones were identical, containing 758 base pairs upstream of the PP5 coding region (261–2, numbers 11 and 15). The third and fourth clones contain 757 and 747 upstream base pairs, respectively. The 758-base pair 5′-flanking region is slightly GC-rich (~57%), and DGBET software (Koto University) was employed to identify putative transcription factor binding sites. Analysis of the 5′-flanking region revealed that a TATA motif was absent (Fig. 2A). The region contains five consensus binding domains for heat shock transcription factors and a single Lyf-1 binding domain. Located ~183 to ~170 upstream of the translation initiation site, there is also a region that encodes a single consensus (36) ERE.

Identification of the Promoter Region of PP5—The promoter activity of the human PP5 gene (PP5) was assessed in MCF-7 cells using PP5-pLucLink-V.2 luciferase reporter plasmids. The 5′-flanking region of human PP5 identified with 5′ RACE (described above) and three sequentially deleted regions of the 5′-flanking region were subcloned into luciferase reporter plasmids and assayed for luciferase activity after transfection into MCF-7 cells. PP5-luc-643 and PP5-luc-184 had marked activi-

2 The pH indicator, phenol red, is structurally reminiscent to estrogen and often contains estrogenic contaminants (32).

3 17β-estradiol levels were measured by clinical chemical analysis (33).
ties, whereas the luciferase activity of PP5-luc-110 was similar to the control Luclink vector (Fig. 2B). These results suggest that the region \(-184 \text{ to } -110\) has basal level promoter activity and that the Lyf-1 and heat shock transcription factor sites identified are not important for basal PP5 expression. Next, the estrogen responsiveness of the reporter plasmids was examined. As seen in Fig. 2, D and E, the activity of reporter plasmids containing EREs (PP5-luc-184 and PP5-luc-643) was enhanced with estrogen treatment. The luciferase assays indicated that a region containing the ERE \((-110 \text{ to } -184)\) is indeed important for transcription activity and the responsiveness to estrogen. To confirm that the ERE identified in the promoter region of PP5 binds to ERs, we next used gel mobility shift analysis using full-length human ER and a double-stranded \(^{32}\)P-labeled DNA probe encoding the ERE identified in the PP5 promoter (PP5-ERE). As seen in Fig. 2C, after electrophoresis on 4–15% polyacrylamide gels a single major signal was detected with the PP5-ERE probe (lane 1). To investigate the specificity of the binding, competition analysis was performed. The addition of a second unlabeled double-stranded ERE probe (sense strand, 5’-GGA TCT AGG TCA GTG TGA CCC CGG ATC-3’) produced a dose-dependent competition with the \(^{32}\)P-labeled PP5-ERE probe (lanes 2 and 3). In contrast, a control probe (sense strand, 5’-GGA TCT AGT ATC AGT GTG ACC CGG GAT C-3) with only two base substitutions in the ERE consensus region (shown in bold) does not compete with the ER-PP5-ERE complex (lane 5). A super shift of the major signal was also produced after the addition of an ER-specific antibody, confirming that the signal was indeed produced by the association of the probe with the ER (data not shown).

**Inhibition of PP5 Expression Suppresses the Growth of MCF-7 Cells**—The data described above suggests that PP5 may participate in estrogen-dependent growth control. Alternatively, increased PP5 expression, although induced by estrogen, may simply accompany rapid cell growth. If increased PP5 expression is necessary for estrogen-dependent cellular proliferation, then the inhibition of PP5 expression should inhibit MCF-7 cell growth even in the presence of estrogen. To test this possibility, we treated MCF-7 cell cultures grown in estrogen-containing FCS with 500 nM ISIS 15534. Because ISIS 15534 acts via RNase H-mediated degradation, it cannot be employed...
FIG. 2. Promoter analysis of the human PP5 gene (PPP5). A, nucleotide sequence of the 5′-flanking region for the human PPP5 gene. The sequence of a 758-base pair region identified by 5′-RACE analysis is shown. Sequences with possible biological significance (consensus heat shock transcription factors (HSF), Lyf-1, and ERE) are shaded and underlined. The codon encoding the initiating methionine (ATG) is indicated in bold type. Arrows indicate the regions used to construct the PP5-luciferase reporter plasmids. B, luciferase-enhancing activities of DNA constructs containing the 5′-upstream region of the PP5 gene. The structures of the PP5-luciferase reporter deletion series of plasmids are shown schematically (left), and their respective luciferase activities are shown to the right of each construct. MCF-7 cells were plated in estrogen-depleted medium as described above for 3 days prior to transfection with the indicated reporter plasmids. The transfection and measurement of luciferase activity were conducted as described under “Experimental Procedures.” Consensus heat shock transcription factors, Lyf-1, and ERE sequences are indicated by ovals, filled rectangles, and squares, respectively. A reporter plasmid lacking the PP5 5′-flanking region (Luclink) was used as a control. C, gel mobility analysis of human ER. Oligonucleotides containing the putative ERE (−191 to −157) identified in the human PP5 promoter were synthesized, labeled, and incubated with 1.4 μg of human estrogen receptor-α. The binding reactions were incubated at room temperature for 25 min. The association of probe with the ERE was then detected by autoradiography after separation on a 4–15% Tris-HCl Criterion gel.
to induce rapid changes in PP5 activity. Rather, Northern analysis indicates that ISIS 15534 takes –4–6 h to enter the cell and effectively induce mRNA degradation. Because of the half-life of the preexisting protein, it takes another ~24 h for human PP5 protein levels to fall (29). Nonetheless, ISIS 15534 potently inhibits the expression of PP5 in cultured cells for ~72 h (IC_{50} of ~50 nm), which affords an ~48-h window to study cells in which the expression of PP5 is essentially ablated (28, 29). Unlike the mismatched control antisense oligonucleotides, which have no apparent effect on PP5 expression or cellular proliferation (28, 29), the change in the slope of the growth curves becomes evident ~24 h after antisense treatment (Fig. 3) suggests that the suppression of PP5 expression inhibits MCF-7 cell growth. To further characterize the role of PP5 in estrogen-dependent growth, MCF-7 cells were placed in estrogen-depleted medium for 3 days and then treated with 10 nM E_2, 100 nM E_2, and ISIS 15534 or a mismatched control oligonucleotide (MM Control). The number of cells in cultures treated with 500 nM ISIS 15534 (•) or 500 nM MM controls (▽) was then determined daily for 2 consecutive days. The time of treatment is indicated by an arrow. Northern blots showing PP5 and GAPDH mRNA levels of cells treated with 500 nM ISIS 15534 are also shown, with day zero representing cellular levels at the time of treatment.

Overexpression of PP5 Enables the Proliferation of ER-dependent MCF-7 Cells in the Absence of Estrogen—Although the inhibition of PP5 expression inhibits MCF-7 cell growth (Figs. 3 and 4), because ISIS 15534 also inhibits the estrogen-independent growth of A549 cells (28), the causal relationship between E_2-stimulated PP5 expression and cell growth was not established. Therefore, to gain further insight into the role(s) of PP5 in estrogen-stimulated tumor cell growth, we derived several double-stable MCF-7 cell lines in which the expression of wild-type PP5 was placed under the control of tetracycline-regulated transactivator and operator plasmids. In these cell lines, tetracycline acts as a suppressor of PP5 gene expression, and upon tetracycline withdrawal the tetracycline-controlled transactivator drives the expression of PP5. Employing Northern blot analysis, we identified cell lines (e.g. MCF-7-PP5 h1) in which the induction of PP5 expression achieved by tetracycline removal was comparable with that produced by treatment with estrogen. When placed in estrogen-deficient medium, ~48 h after tetracycline withdrawal PP5 expression increased ~2-fold (Fig. 5A). Western analysis revealed a similar increase in PP5 protein levels. This increase in PP5 expression can be maintained for at least five passages, and shortly after the induction of PP5 expression the MCF-7-PP5 h1 cells gain the ability to proliferate in estrogen-deficient medium, with growth rates comparable with those observed after treatment with E_2. Several additional MCF-7-PP5-inducible cell lines (derived in different experiments and from different dishes from that which

(Bio-Rad) as described under “Experimental Procedures.” Lane 1, PP5-ERE, labeled nucleotide sequence –191 to –157 from the human PP5 5′-flanking region; lanes 2 and 3, excess cold probe controls, samples treated in an identical manner to that of lane 1 after incubation in the presence of 90 ng (Lane 2) and 120 ng (Lane 3) of a nonradioactive consensus ERE probe; lane 4, blank; lane 5, negative cold probe control, samples treated in an identical manner to that of lane 1 after incubation in the presence of 120 ng of a nonradioactive probe identical to that used in lanes 2 and 3 except for three base substitutions in the ERE consensus region (the sequences of the probes are shown under “Results”). D and E, estrogen responsiveness of the human PP5 promoter. Luciferase reporter plasmids were transfected into MCF-7 cell cultures that were maintained in estrogen-depleted medium for 3 days prior to the transfection. The cell cultures were then treated with 10 nM E_2, (+) Estrogen) or vehicle (−) Estrogen) and incubated for 24 h. Luciferase activity was measured as described under “Experimental Procedures” and plotted as a ratio of luciferase activity to total protein (D) or luciferase to Renilla activity. The data are presented as the mean ± S.E. The statistical significance is designated by * (p < 0.005).
PP5 Converts MCF-7 Cells into an Estrogen-independent Phenotype

PP5 expression in combination with the selection of a spontaneously occurring mutation during the selection for cells with tetracycline-regulated PP5 expression. Cell lines expressing >2.5-fold induction of PP5 could not be established, suggesting that a marked increase in PP5 expression was harmful.

Treatment with ISIS 15534 Has No Apparent Effect on E2-induced Expression of c-myc or cyclin D1—The ability of estrogen to stimulate proliferation of human cells has been studied extensively. Such studies have revealed that estrogen-dependent growth is initiated by the binding of estrogen to its cognate intracellular receptor. Agonist binding activates the ER complex, which in turn induces the transcription of ER-responsive genes (i.e. c-myc and cyclin D1) that facilitate G1/S phase progression. Once activated, Cyclin D1 is capable of promoting the response in the absence of hormone via the direct association/activation of the ER (37, 38). To gain further insight into the mechanism(s) by which increased PP5 expression facilitates cell growth, we examined the relationship between PP5 and events associated with estrogen-dependent growth. The Northern blot analysis of RNA obtained from ISIS 15534-treated cells was examined to determine whether the inhibition of PP5 expression affects the expression of these key regulators of estrogen-induced proliferation. These studies revealed that although the inhibition of PP5 expression inhibits estrogen-stimulated MCF-7 cell proliferation, treatment with up to 500 nM ISIS 15534 resulted in no apparent effect on estrogen-induced expression of c-myc or cyclin D1 mRNA (Fig. 6). In addition, the induction of PP5 expression, evident by ~12 h, occurs subsequent to the induction of c-myc and cyclin D1, which are evident by ~1–2 and ~6–8 h, respectively (data not shown). Thus, although PP5 expression seems critical for MCF-7 cell growth, PP5 likely acts either downstream or independently of c-Myc and Cyclin D1.

DISCUSSION

Mammalian PP5 contains a catalytic domain with shared homology to the PP1/PP2A/PP4 family of PPases (25, 27) and an extended amino-terminal domain that contains three tetratricopeptide motifs (25, 27). In vitro, proteolysis leading to the removal of the tetratricopeptide domain produces a marked increase in activity, suggesting that the tetratricopeptide-containing domain has autoinhibitory activity (27). PP5 activity is also enhanced by the addition of polyunsaturated lipids in vitro (27, 39). However, because of the high concentrations of lipids needed for activation, it is not yet clear whether lipid activation is physiologically important. PP5 has been shown to co-precipitate with that produced by the addition of 10 nM 17β-estradiol (+) or a vehicle (−) to replicate under estrogen-deficient conditions seems to arise from enhanced PP5 expression alone and not from enhanced repression of PP5 expression in combination with the selection of a spontaneously occurring mutation during the selection for cells with tetracycline-regulated PP5 expression.

Figure 5. Constitutive overexpression of PP5 in MCF-7 cells enables cellular proliferation in the absence of estrogen. Double-stable cell lines were developed by the stable transfection of MCF-7 Tet-off cells with a pBI-epidermal growth factor-derived plasmid encoding full-length human PP5. Clones were grown in the presence of 2 µg/ml tetracycline (to suppress PP5 expression) and selected for by resistance to G418. A, one cell line, MCF-7-PP5 h1, in which the level of PP5 mRNA expressed upon the removal of tetracycline was comparable with that produced by the addition of 10 nM 17β-estradiol (+) to the parental MCF-7 cells. Inset, a representative Northern and Western blot comparing the level of PP5 mRNA and protein, respectively, in cultures grown with and without tetracycline. −, mRNA from MCF-7-PP5 h1 cells grown in the presence of 2 µg/ml tetracycline; −, mRNA from cell cultures 48 h after tetracycline withdrawal; 1, PP5 protein from cell cultures on day 1; 4, PP5 protein from cell cultures on day 4. Once induced by tetracycline withdrawal, PP5 mRNA levels remained constant (data not shown). Assessment of cell growth over a period of 15 days in estrogen-deficient phenol red-free DMEM medium supplemented with 10% gelded horse serum revealed that, unlike control parental MCF-7 cells, MCF-7-PP5 h1 (data not shown). Assessment of cell growth over a period of 15 days in estrogen-deficient phenol red-free DMEM medium supplemented with 10% gelded horse serum revealed that, unlike control parental MCF-7 cells, MCF-7-PP5 h1, and MCF-7-PP5 k1 (27), which remained static, the removal of tetracycline (indicated by an arrow) afforded MCF-7-PP5 h1 cell cultures the ability to proliferate without estrogen supplementation (C). At day 6, the cells overexpressing PP5 reached confluency, and both cell lines were split and newly seeded (indicated by d). To ensure low estrogen conditions at the start of the experiment, cells were maintained in phenol red-free DMEM with 10% gelded horse serum for 3 days prior to the experiment. B, growth rates of PP5-Tet-off cell lines. The growth rates of three double-stable cell lines developed by the stable transfection of MCF-7 Tet-off cells with a pBI-derived plasmid encoding full-length human PP5/MCF-7-PP5 h1 ( ), MCF-7-PP5 k1 ( ), MCF-7-PP5 l1 ( ), and a control plasmid lacking PP5 ( ) are shown. To ensure estrogen depletion, the cells were plated in estrogen-depleted medium for 24 h prior to the replacement of medium that lacked tetracycline (day 3). Western analysis revealed that the removal of tetracycline resulted in a 2.4-, 1.4-, and 1.2-fold induction of PP5 protein levels that reached a maximum by day 4 for MCF-7-PP5 h1 ( ), MCF-7-PP5 k1 ( ), and MCF-7-PP5 l1 ( ), respectively. In contrast, an induction was not observed in control cells ( ). For both graphs the data are representative of at least three separate experiments, and each point represents the mean of triplicate cultures with error bars representing the S.E.

Figure 6. Estrogen-induced expression of c-myc and cyclin D1 does not depend on PP5. MCF-7 cells were placed in estrogen-deficient phenol red-free DMEM medium for 3 days. The cell cultures were then treated with 500 nM ISIS 15534 (+) or a control (−) as described in the Fig. 3 legend. After 16 h, 10 nM 17β-estradiol (+) or a vehicle (−) was added to the individual cell cultures as indicated. Twenty-four h after estradiol treatment, total RNA was isolated and PP5, c-myc, cyclin D, and GAPDH mRNA levels were assessed by Northern blot analysis. The autoradiographs shown are representative of at least three experiments.
PP5 Converts MCF-7 Cells into an Estrogen-independent Phenotype

The protein product of \( e_{2} \)-mediated proliferation is associated with increased \( c-myc \) expression, the expression of \( cyclin D1 \) is indirect, requiring \( de novo \) synthesis of an as yet unidentified intermediate protein(s) (48, 49). In MCF-7 cells, \( e_{2} \)-mediated proliferation is associated with increased \( cyclin D1 \) expression, the formation of \( Cyclin D1/Cdk4 \) complexes, and the activation of both \( Cyclin D1/Cdk4 \) and \( Cyclin E/Cdk2 \) complexes (48–51). The overexpression of \( cyclin D1 \) is sufficient to overcome SERM-induced \( G_{1} \) growth arrest, and the \( Cdk4 \)-inhibitor, p16INK4A, as well as antibodies targeting \( Cyclin D1 \) prevent \( e_{2} \)-induced \( G_{1} \)-S phase progression after microinjection into breast cancer cells (52, 53). In addition, overexpression of \( cyclin D1 \) activates ligand-free ER and stimulates ligand-bound ER by forming a complex with the steroid receptor and enhancing its transcriptional activity (37, 38). These observations support the theory that \( Cyclin D1 \) mediates \( e_{2} \)-induced proliferation. However, some estrogen-dependent processes such as normal mammary gland development occur in mice carrying null mutations in both \( cyclin D1 \) alleles (54–56), arguing that \( e_{2} \)-stimulated proliferation may also occur via \( Cyclin D1 \)-dependent processes. As observed with \( c-myc \), the inhibition of PP5 expression had no apparent effect on \( e_{2} \)-induced \( cyclin D1 \) expression. Thus, again, the growth-regulating actions of PP5 seem independent of or reside downstream from \( Cyclin D1 \).

Although the substrates for PP5 are not yet known, in MCF-7 cells we found no evidence that PP5 influences ER-mediated transcriptional activation or other growth-promoting actions leading to the induction of \( c-myc \) or \( cyclin D1 \). In addition, \( e_{2} \)-induced PP5 expression is most evident –12 h after treatment with \( e_{2} \), which is well after the induction of \( c-myc \) and \( cyclin D1 \) (evident 6–8 h after treatment with \( e_{2} \)). In other cell lines, the suppression of PP5 expression enhances the transcriptional activity of p53 (28) and/or ligand-activated glucocorticoid receptors (29). Both p53 and GR are well established as key regulators of growth suppression, with the activation of GR or p53 alone sufficient to arrest the growth of many human cells in culture. In addition, studies conducted in ovariectomized rats treated with \( e_{2} \) suggest glucocorticoids also have a direct effect on the target tissues of sex steroids, with stress levels of glucocorticoids blocking estradiol-mediated uterine growth (57). Because human cells are continuously exposed to glucocorticoids \textit{in vivo} and most exhibit basal p53 expression, \( e_{2} \)-induced PP5 expression may aid growth by suppressing GR- and/or p53-dependent signaling cascades that restrict cell growth. Such a concept is supported by transient transfection studies conducted in A549 cells in which ISIS 15534-mediated suppression of PP5 expression enhances dexamethasone-induced reporter plasmid activity by –10-fold (29). We observed a similar although smaller (–2-fold) effect in MCF-7 cells. However, because dexamethasone did not suppress MCF-7 cell growth, further studies will have to be conducted to determine the precise role of PP5 in steroid hormone-mediated growth control. Nonetheless, the observation that constitutive expression of PP5 relieves human MCF-7 breast carcinoma cells from their dependence on estrogen for growth suggest that aberrant PP5 expression may contribute to outgrowth of tumors refractory to the antiestrogenic effects of SERMs. Because the effective options available to physicians for the treatment of breast cancer after the re-emergence of...
antiestrogen-resistant metastases are currently inadequate, the possible role of PP5 in "acquired antiestrogen resistance" is clearly worthy of further investigation.

Acknowledgments—We thank Dr. Ray Hester for FACS analysis and Jamie Koons for excellent technical assistance.

REFERENCES
1. International Agency for Research on Cancer (1992) Publication no. 120 (World Health Organization)
2. S. R. Scott, J. A., McGuire, W. L. (1991) in Endocrine-dependent Tumors (Voight, K. D., and Knabbe, C., eds) pp. 179–196, Raven Press, Ltd., New York
3. Lippman, M., Bolan, G., and Huff, K. (1976) J. Cell Sci. 21, 587–608
4. Sutherland, R. L., Reddel, R. R., and Green, M. D. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3642–3646
5. Ptashne, M. (1988) Nature 335, 683–689
6. Ptashne, M. (1989) Science 245, 371–378
7. Osborne, C. K. (1992) J. Natl. Cancer Inst. 84, 2038–2048
8. Osborne, C. K. (1993) J. Cell. Biochem. 50, 848–855
9. Osborne, C. K., Elledge, R. M., and Fuqua, S. A. W. (1996) Endocrinology 133, 1519–1523
10. Katzenellenbogen, J. A., and Honkanen, R. E. (1999) J. Biol. Chem. 274, 6366–6372
11. Katzenellenbogen, J. A., and Honkanen, R. E. (1999) J. Biol. Chem. 274, 6366–6372
12. Katzenellenbogen, J. A., and Honkanen, R. E. (1999) J. Biol. Chem. 274, 6366–6372
13. Katzenellenbogen, J. A., and Honkanen, R. E. (1999) J. Biol. Chem. 274, 6366–6372
14. Katzenellenbogen, J. A., and Honkanen, R. E. (1999) J. Biol. Chem. 274, 6366–6372
Identification of an Estrogen-inducible Phosphatase (PP5) That Converts MCF-7 Human Breast Carcinoma Cells into an Estrogen-independent Phenotype when Expressed Constitutively

Gudrun Urban, Teresa Golden, Ileana V. Aragon, Jonathan G. Scammell, Nicholas M. Dean and Richard E. Honkanen

J. Biol. Chem. 2001, 276:27638-27646. doi: 10.1074/jbc.M103512200 originally published online April 30, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103512200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 54 references, 27 of which can be accessed free at http://www.jbc.org/content/276/29/27638.full.html#ref-list-1