Variant estrogen receptor- α messenger RNA expression in hormone-independent human breast cancer cells

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ABSTRACT

T5-PRF cells are insensitive to the growth-stimulatory effects of estrogen while still retaining expression of estrogen receptor- α (ER- α). In the apparent absence of ligand, T5-PRF cells have a 3.6 ± 0.5 (S.E.M.)-fold increased basal ER- α activity and elevated basal progesterone receptor levels compared with the parent, estrogen-sensitive, T5 cells. Long-range ER- α reverse transcription-PCR was performed to characterize variant ER- α mRNA expression in the two cell lines. An increased relative expression of an exon 3/4-deleted ER- α mRNA variant was found in T5-PRF. Recombinant expression of this ER- α variant resulted in

significantly increased estrogen responsiveness, as well as a trend to increased basal ligand-independent activity when expressed with wild-type ER- α in ER-negative cell lines, as well as significantly increasing both ligand-independent and estrogen-induced ER- α transcriptional activity when expressed in parental T5 cells. These results suggest a role for altered variant ER- α in ligand-independent activation of ER- α which may contribute to hormone independence in breast tumors.

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INTRODUCTION

Breast cancer is a hormonally responsive cancer and hormones, including estrogen, are required for breast cancer growth (Dickson 1991). Estrogens promote the growth of human breast cancer, and as such, most endocrine therapies are aimed at blocking the growth-promoting effects of estrogen (e.g. antiestrogen such as tamoxifen). Breast cancers are classified according to their requirement for proliferation as being either hormone dependent or hormone independent, based ultimately on the response to endocrine therapy of metastatic disease (Nandi *et al.* 1995). The level of estrogen receptor- α (ER- α) in human breast cancer is used as a marker not only of potential therapeutic response to endocrine therapy, but as a marker of prognosis and survival (Merkel & Osborne 1989).

The evolution of breast cancer into an estrogen-independent growth phenotype is thought to be an important step in the progression of breast cancer to hormone independence and endocrine therapy resistance (Clarke *et al.* 1990, Leonessa *et al.* 1992). Understanding the factors that contribute to the

development of a hormone-independent phenotype is of major importance in terms of breast cancer therapeutics. Resistance to endocrine therapies may be due to a number of factors. In some cases, hormone independence and resistance can occur due to loss of ER expression, but most tumors that have developed resistance to endocrine therapy remain receptor positive (Horwitz 1993).

Several breast cancer cell lines in culture also require estrogen for growth, and long-term culture in estrogen-depleted conditions can result in cells becoming apparently independent of the requirement for estrogen for growth. Indeed, the development of estrogen-independent growth in human breast cancer is thought to be one of the initial steps in the progression to hormone independence and resistance to endocrine therapies (Leonessa *et al.* 1992). However, the mechanisms responsible for the development of estrogen independence in the presence of continued expression of ER- α are poorly understood. In order to address this we have developed a breast cancer cell model of apparent estrogen independence (Coutts *et al.* 1996). T5 human breast cancer cells are ER- α positive and

estrogen treatment in culture results in increased proliferation of these cells. An estrogen-nonresponsive cell line, T5-PRF, was developed from T5 cells by chronically depleting the cells of estrogen in long-term culture. These cells are insensitive to the growth-stimulatory effects of estrogen seen in the parent cell line while still retaining expression of the ER- α (Coutts *et al.* 1996). However, these cells remain sensitive to the growth inhibitory effects of 4-hydroxy-tamoxifen (OT) and ICI 164,384 (ICI), although they have reduced sensitivity to ICI compared with the parent T5 cells (Coutts *et al.* 1996).

We have investigated the ligand-dependent and -independent activity of the endogenous ER- α as well as the pattern and potential function of ER- α variant expression in T5 and T5-PRF human breast cancer cells.

MATERIALS AND METHODS

Materials

(32 P)dCTP and (35 S)ATP were purchased from ICN (St-Laurent, Quebec, Canada). Dulbecco's Minimal Essential Medium (DMEM) powder and fetal bovine serum were purchased from GIBCO/BRL (Burlington, Ontario, Canada). Horse serum and epidermal growth factor (EGF) were purchased from UBI (Lake Placid, New York, NY, USA). All other cell culture ingredients were purchased from Flow Laboratories (Mississauga, Ontario, Canada). Cholera toxin, OT, estradiol-17 β (E2) and dexamethasone were obtained from Sigma Chemical Co. (St Louis, MO, USA). [14 C]Chloramphenicol, [3 H]R5020 (88.7 Ci/mmol), [35 S]methionine and R5020 were obtained from NEN (Lachaire, Quebec, Canada). ICI was a gift from ICI (Macclesfield, Cheshire, UK).

Cells and cell culture

T5 cells, previously called T-47D5, were originally thought to be a T-47D subline; however, DNA fingerprinting analysis showed that they were an MCF-7 subline (Watts *et al.* 1992). T5 and MDA-MB-231 human breast cancer cells were routinely cultured in DMEM containing 5% v/v fetal calf serum, 1% w/v glucose, glutamine and penicillin-streptomycin. T5-PRF cells were routinely cultured in phenol red-free DMEM supplemented with 5% v/v twice charcoal-dextran-stripped fetal calf serum and 1% w/v glucose, glutamine, and penicillin-streptomycin (PRF/DMEM). MCF10A1 human breast epithelial cells (Karmanos Cancer Institute, Detroit, MI, USA)

were grown routinely in DMEM containing 5% v/v horse serum, 1% w/v glucose, glutamine and penicillin-streptomycin, 0.1 μ g/ml cholera toxin, 20 ng/ml human EGF (hEGF), 10.4 μ g/ml bovine insulin and 1 μ M hydrocortisone (DMEM-special). Transient transfections and steroid receptor assays were performed in PRF/DMEM. Transient transfections using MCF10A1 cells were performed in phenol red-free DMEM containing 5% v/v charcoal-stripped horse serum, 1% w/v glucose, glutamine and penicillin-streptomycin (PRF/DMEM-hs) and cells were passaged once prior to transfection in phenol red-free DMEM containing 5% v/v charcoal-stripped horse serum, 1% w/v glucose, glutamine, penicillin-streptomycin, 0.1 μ g/ml cholera toxin, 20 ng/ml hEGF, 10.4 μ g/ml bovine insulin and 1 μ M hydrocortisone (PRF/DMEM-special).

Progesterone receptor (PR) assays

PR assays were performed using whole-cell ligand-binding assays as previously described (Murphy & Dotzlaw 1989a). [3 H]R5020 and [3 H]5020 plus 100-fold molar excess unlabeled R5020 were used to determine PR total and nonspecific binding respectively. All assays were performed in the presence of 100 nM dexamethasone to prevent binding of R5020 to the glucocorticoid receptor.

Transient transfections and chloramphenicol acetyltransferase (CAT) assays

T5, T5-PRF and MDA-MB-231 cells were passaged once in PRF/DMEM and set up in 100 mm diameter dishes at 0.5×10^6 cells per dish in PRF/DMEM the day before transfection. MCF10A1 cells were passaged once in PRF/DMEM-special and set up in 100 mm diameter dishes at 2×10^6 cells per dish in PRF/DMEM-special 2 days before transfection. The following day the medium was changed to PRF/DMEM-hs and cells were transfected the following day, using the calcium phosphate/glycerol shock method (Graham & Van der Eb 1973) using an equal volume $2 \times$ BBS buffer (50 mM N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM Na₂HPO₄, pH 6.95), followed by a 2 min glycerol shock (20% v/v). Cells were washed twice with PBS and given fresh medium plus or minus 10 nM E2, 10 nM E2 plus 1 μ M ICI or 1 μ M ICI alone. After 24 h of treatment, the cells were harvested, cell extracts prepared and CAT activity measured (Kingston 1989). Transfection efficiency was determined by cotransfection of pCH110 (β -galactosidase expression vector; Pharmacia, Baie

d'Urfé, Québec, Canada) and assay of β -galactosidase activity (Rosenthal 1987) was used to correct volumes of cell extracts used for CAT assay. Therefore all results are expressed as percent CAT activity or fold CAT activity after correction for transfection efficiency. T5 and T5-PRF cells were transfected with 5 μ g estrogen response element (ERE)-thymidine kinase (tk)-CAT (Seiler-Tuyns *et al.* 1986), to determine ER- α transcriptional activity, along with 5 μ g pCH110. In the experiments where activity of exon 3/4-deleted ER- α (d3/4) was examined in T5 cells, transfections were performed using 5 μ g ERE-tk-CAT, 5 μ g pCH110 plus or minus d3/4 expression vector (0.1–1 pmol) or vector DNA alone. MDA-MB-231 and MCF10A1 cells were transfected with 5 μ g ERE-tk-CAT, 5 μ g pCH110, plus or minus 0.5 pmol HEGO (an expression plasmid containing wild-type ER- α coding region cloned into the eukaryotic expression vector pSG5, a gift from Dr P Chambon, Strasbourg, France (Tora *et al.* 1989a)) with increasing amounts (0.5–2 pmol) of d3/4 expression vector or vector DNA alone.

Long-range ER- α reverse transcriptase (RT)-PCR

Total RNA was extracted (Trizol reagent, GIBCO/BRL, Grand Island, New York, NY, USA) and reverse transcribed as described previously (Leygue *et al.* 1996c). The primer pair used consisted of 1/8U primer (5'-TGCCCTACTACCTGGAGA ACG-3', sense; located in WT-ER- α exon 1; nucleotides 615–637) and 1/8L primer (5'-GCC TCCCCGTGATGTAA-3', antisense; located in WT-ER- α exon 8; nucleotides 1995–1978). Nucleotide positions given correspond to published sequences of the human ER- α cDNA (Green *et al.* 1986). PCR amplifications were performed as previously described (Leygue *et al.* 1996a). PCR products were separated on 3.5% polyacrylamide gels containing 7 M urea, gels dried and labeled products visualized by autoradiography. PCR products were subcloned and sequenced as previously described (Leygue *et al.* 1996c).

Construction of variant ER- α expression vector

The RT-PCR product corresponding to the d3/4 cDNA was cloned into the TA cloning vector (Invitrogen TA cloning kit, Carlsbad, CA, USA). StuI digestion of this plasmid released an exon 3/4-deleted fragment which was used to replace the corresponding region of wild-type ER- α from pOR8 (Tora *et al.* 1989a) (contains a glycine to valine

point mutation at amino acid 400). Stu I sites are in exon 2 and 7 of wild-type ER- α and the subcloned d3/4 PCR fragment resulted in a correction to the wild-type sequence of glycine at amino acid 400 (in exon 5 in pOR8). The full-length EcoRI ER- α fragment from HEGO was then excised and replaced with the corresponding fragment from pOR8 containing the d3/4 cDNA. The identity of the expression plasmid containing the d3/4 was confirmed by restriction enzyme digest and sequence analysis.

In vitro transcription and translation

In vitro transcription/translation reactions were performed using a coupled transcription/translation system (TnT coupled Reticulocyte Lysate System, Promega, Madison, WI, USA). Reactions were performed according to the manufacturer's instructions.

Western blotting and immune detection

Whole-cell extracts (dissolved in 8 M urea) were analyzed using 10% SDS-PAGE with a 4% stacking gel at 200 V for 45 min at room temperature according to the Laemmli method (Laemmli 1970). Gels were transferred to nitrocellulose using CAPS transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), pH 11, 20% methanol) and transferred for 1 h at 120 V at 4 °C. Blots were blocked for 1 h at room temperature in 5% skimmed milk/Tris-buffered saline containing 0.5% Tween-20. Blots were incubated with either ER- α -specific primary antibody, H226 (a generous gift from Dr G Greene, University of Chicago, IL, USA), which recognizes an epitope in exon 1/exon 2 region of the wild-type ER- α , or the ER- α -specific antibody, AER 308 (Neomarkers, Fremont, CA, USA), which recognizes an epitope in exon 4 of the wild-type ER- α , overnight at 4 °C in 1% skimmed milk/Tris-buffered saline containing 0.5% Tween-20. Blots were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature in 1% skimmed milk/Tris-buffered saline containing 0.5% Tween-20. Detection was carried out using an enhanced chemiluminescence detection system according to the manufacturer's instructions (Amersham International, Amersham, Bucks, UK).

Statistical analysis

Statistical analyses of ER- α transcriptional activity and PR levels were performed using paired Student's *t*-test. Statistical analysis on the effects of

d3/4 on ERE-tk-CAT activity in MDA-MB-231 and MCF10A1 cells was done by ANOVA (two-tailed). Statistical analysis on the effects of d3/4 on ERE-tk-CAT activity in T5 cells was done by ANOVA (two-tailed) after log transformation of the data. Statistical analyses were performed with the help of M Cheang, University of Manitoba, Biostatistical Consulting Unit.

RESULTS

Apparent ligand-independent (basal) activity of ER- α is increased in T5-PRF cells

Previously, we have shown that T5-PRF cells (derived by long-term passage of parental T5 cells in estrogen-depleted medium) are no longer growth responsive to estrogen in culture, but still retain expression of ER- α (Coutts *et al.* 1996). To investigate further the mechanism(s) responsible for estrogen nonresponsiveness in these cells, ER- α transcriptional activity was examined by transient transfection assays using an estrogen-responsive reporter gene. Figure 1A represents the fold difference in CAT activity between T5 and T5-PRF cells. As expected, estrogen treatment increases CAT activity in T5 and to a lesser extent in T5-PRF cells, while the antiestrogen ICI inhibits the estrogen-induced transcriptional activity of the ER- α in both cell lines. In the absence of added estrogen there is a low basal ER- α activity in parental T5 cells; however, in the estrogen-nonresponsive T5-PRF cells, the basal ER- α activity was 3.6 ± 0.5 -fold (mean $\pm$ S.E.M., $n=7$) higher than that seen in T5 cells ($P>0.05$). Consistent with the increased basal CAT activity in T5-PRF cells being mediated by ER- α , treating cells under basal conditions with ICI alone almost completely abolished the basal transcriptional activity measured (Fig. 1B).

PR levels are elevated in T5-PRF cells

PR expression is a marker of ER- α activity (Horwitz & McGuire 1978), therefore we examined PR levels to determine if the increase in basal ER- α activity in T5-PRF cells was reflected in an endogenous estrogen-responsive gene. Under basal (i.e. no added estrogen) conditions the T5-PRF cells have significantly higher PR levels (~ 3 -fold) than the parent T5 cells passaged twice in estrogen-depleted medium (PRF/DMEM as defined in Materials and Methods) before receptor assays (464 ± 20 fmol/ 10^6 cells vs 148 ± 40 fmol/ 10^6 cells, mean $\pm$ S.E.M., $n=3$, see Fig. 2). Previously, we had shown that T5-PRF

FIGURE 1. ER transcriptional activity. (A) T5 and T5-PRF cells were transfected and CAT assays performed as described in Materials and Methods. Results are expressed as fold CAT activity compared with T5 basal (arbitrarily set at 1.0). * $P<0.05$, Student's *t*-test (compared with T5 basal). Results represent mean $\pm$ S.E.M., $n=7$. (B) T5-PRF cells were transfected and CAT assays performed as described in Materials and Methods. Results represent percent CAT activity as compared with T5-PRF basal set at 100%, mean $\pm$ range, $n=2$.

cells retained expression of ER- α which was approximately 50% of the levels seen in the parent T5 cells, as determined by a ligand-binding assay (Coutts *et al.* 1996). Since we observed increased basal activity from both an estrogen-responsive reporter gene and an endogenous estrogen-responsive gene (PR) in T5-PRF cells despite a decreased level of endogenous ligand-binding ER, we reasoned that the intrinsic activity of the wild-type ER in these cells was increased or some

FIGURE 2. PR levels. PR levels were determined by whole-cell binding as described in Materials and Methods. PR levels are expressed as fmol PR/10⁶ cells and results represent mean $\pm$ S.E.M., $n=3$. ** $P<0.01$, Student's t -test.

ER-like activity existed that was not detected by ligand-binding experiments.

Expression of a variant ER- α mRNA deleted in exons 3 and 4 is increased in T5-PRF cells

Alterations in the structure or presence of variant forms of the ER- α with ligand-independent activity could be one mechanism for our observed results. Long-range ER- α RT-PCR analysis (Leygue *et al.* 1996a) was performed on RNA isolated from T5 and T5-PRF cells to examine the pattern of deleted variant ER- α mRNA expression. RT-PCR analysis was performed, using a primer pair specific for exons 1 and 8 of the wild-type human ER- α sequence, allowing detection of any variant ER- α mRNA species containing both exons 1 and 8 of wild-type ER- α sequence. Figure 3 shows the PCR products obtained and the presence of a 928 bp band whose relative expression is markedly increased in T5-PRF estrogen-independent cells. To confirm the identity of this variant, the cDNA corresponding to the 928 bp band was subcloned

FIGURE 3. Identification of d3/4 mRNA. (A) Long-range ER- α RT-PCR. Total RNA was extracted from T5 and T5-PRF cells, reverse transcribed and PCR amplified using 1/8U and 1/8L primers. Labeled PCR products were separated on 3.5% acrylamide-urea gels and visualized using autoradiography. WT-ER=wild type ER- α , D7-ER=exon 7-deleted ER- α , D4-ER=exon 4-deleted ER- α and D3/4-ER=exon 3 and 4-deleted ER- α (d3/4), based on size compared with labeled markers (not shown). (B) Sequence of d3/4 cDNA. The 928 bp PCR product was excised from a gel and subcloned (in triplicate) and three colonies from each independent subcloning were sequenced to confirm the identity of d3/4 cDNA.

FIGURE 4. *In vivo* expression of d3/4 protein. MCF10A1 human breast epithelial cells were transfected with the appropriate expression vectors, cells were lysed in 8 M urea and 10 μ g protein run on a 10% SDS-acrylamide gel. Gels were transferred to nitrocellulose and Western blotting performed using ER- α Ab H226. Lane 1: control cells transfected with 5 μ g of vector (pSG5) alone; lane 2: cells transfected with 5 μ g HEGO (WT-ER- α) expression vector; lane 3: cells transfected with 5 μ g d3/4 expression vector; lane 4: *in vitro*-transcribed/translated WT-ER- α (1 μ l); lane 5: *in vitro*-transcribed/translated d3/4 (2 μ l).

and sequenced. The nucleotide sequence of the cDNA was found to represent a variant ER- α mRNA containing a deletion of both exons 3 and 4. This d3/4 is in frame and is predicted to encode a protein of 443 amino acid residues with a predicted molecular mass of $\sim$ 49 kDa. This putative ER- α -like protein would be missing the second zinc finger of the ER- α DNA-binding domain (DBD), the hinge region and part of the ligand-binding domain.

The exon 3/4-deleted ER- α -like protein increases basal and estrogen-regulated wild-type ER- α transcriptional activity

To address the potential function of this variant ER- α mRNA eukaryotic expression vectors containing d3/4 cDNA were constructed and shown to express a protein of the appropriate size that was recognized by the ER- α antibody H226 that recognizes an epitope encoded in exon 1/2 (A/B region) of wild-type ER- α (data not shown). Using an antibody that recognizes an epitope encoded in exon 4 of the wild-type ER- α the band corresponding to the d3/4 protein is not seen, while wild-type ER- α is still detected (data not shown). Ligand-binding analysis of the *in vitro*-translated d3/4 protein showed little or no ability to specifically bind radiolabeled E2 or OT (data not shown). This protein is missing the second zinc finger of the DBD and as such would not be expected to bind to DNA. We found that under conditions in which *in vitro*-transcribed/translated wild-type ER- α could bind to an oligonucleotide containing the vitellogenin B1 ERE, d3/4 did not demonstrate any specific DNA binding in gel mobility shift assays (data not shown).

To examine the transcriptional activity of the d3/4 variant, transient transfections using ER-negative cell lines were carried out. Under conditions in which transiently transfected wild-type ER- α was transcriptionally active and able to induce CAT activity in a ligand-dependent fashion, the d3/4 did not demonstrate any transcriptional activity on its own (data not shown). This is unlikely to be due to low levels of expression of this transgene, since after transfection of 5 μ g d3/4 vector into MCF10A1 cells we were able to detect a protein corresponding in size to the expected d3/4 protein (Fig. 4). To determine if d3/4 and wild-type ER- α could interact to influence transcription, cotransfections of wild-type ER- α and d3/4 into MDA-MB-231 and MCF10A1 ER-negative breast epithelial cell lines were carried out (Fig. 5A and B). HEGO transfected alone showed the expected estrogen-dependent activity (Fig. 5A and B) while d3/4 alone had no transcriptional activity. However, when increasing amounts of d3/4 were cotransfected with a constant amount of HEGO into MDA-MB-231 cells, d3/4 significantly increased estrogen-dependent activity of wild-type ER- α (ANOVA, $P < 0.0001$). Furthermore, although it did not reach statistical significance there was a trend of increased d3/4 to increase the basal level of activity of the wild-type ER- α . This pattern of activity was also seen when similar experiments were carried out in MCF10A1 cells. A significant increase in the estrogen-dependent activity was seen (ANOVA, $P < 0.05$), with a trend towards increased basal activity.

We next examined the effects of introducing the d3/4 into the parental T5 cells. Transient transfection of d3/4 into T5 cells was carried out and ER- α transcriptional activity measured. Figure 6 shows

the results, and demonstrates that increasing amounts of d3/4 transiently transfected into T5 cells were associated with a significant increase in CAT activity in both the presence and absence of added estrogen (ANOVA, $P<0.05$ and $P<0.0001$ respectively) despite the fact that this variant ER- α

FIGURE 6. Transient transfection of d3/4 expression vector into T5 cells. Cells were grown in PRF-DMEM as described in Materials and Methods and transfected with 5 μ g ERE-tk-CAT expression vector, 5 μ g pCH110 along with the appropriate amount of d3/4 expression vector. Cells were treated with vehicle or 10 nM E2 for 24 h, harvested and CAT assays performed. Results represent mean $\pm$ S.E.M., $n=3-5$, ***b= $P<0.0001$, ANOVA, compared with control basal ERE-tk-CAT activity, *e= $P<0.05$, ANOVA, compared with control E2-treated ERE-tk-CAT activity.

does not bind appreciably to ligand *in vitro* nor has transcriptional activity of its own at this concentration.

DISCUSSION

Numerous studies have identified variant ER- α mRNAs in both normal and neoplastic breast tissue and cell lines (Garcia *et al.* 1988, Murphy & Dotzlaw 1989b, Fuqua *et al.* 1992, McGuire *et al.*

FIGURE 5. Activity of d3/4 in ER- α -negative cells. (A) MDA-MB-231 cells were transfected with 5 μ g ERE-tk-CAT, 1 μ g pCH110, 0.5 pmol HEGO $\pm$ 0.5–2 pmol d3/4 $\pm$ vector DNA to give a total of 17 μ g DNA/dish. Cells were treated with 10 nM E2 for 24 h or vehicle alone as control. Results are expressed as fold CAT activity compared with basal HEGO activity arbitrarily set as 1. Columns represent mean $\pm$ S.E.M., $n=6-7$. ***e= $P<0.0001$ by ANOVA compared with E2-treated HEGO alone. (B) MCF10A1 cells were similarly transfected. Columns represent mean $\pm$ S.E.M., $n=4$. *e= $P<0.05$, ANOVA, compared with E2-treated HEGO alone.

1992, Leygue *et al.* 1996a,c). While still a controversial topic, evidence is emerging to support the existence of ER- α variant proteins, which could correspond to some ER- α variant mRNAs, in some cell lines and tissues *in vivo* (Raam *et al.* 1988, Marigliante *et al.* 1991, Monchamont *et al.* 1991, Scott *et al.* 1991, Fuqua *et al.* 1992, Castles *et al.* 1993, Montgomery *et al.* 1993, Pink *et al.* 1995).

However, the pathophysiological significance of ER- α variant expression is unclear. Altered expression of some ER- α variant mRNAs was found associated with both breast tumorigenesis and breast cancer progression (Fuqua *et al.* 1992, Daffada *et al.* 1995, Murphy *et al.* 1995, Leygue *et al.* 1996b,c). Several studies, using transient transfection analyses, have shown that individual ER- α variant proteins can have both positive and negative effects on wild-type ER- α activity (Fuqua *et al.* 1991, 1992, Wang & Miksicek 1991, Castles *et al.* 1993, Pink *et al.* 1995, 1996, Rea & Parker 1996, Desai *et al.* 1997). Conflicting results for some ER- α variants have been obtained (Wang & Miksicek 1991, Fuqua *et al.* 1993), which may be due to cell- and promoter-specific events previously identified for various structure/function domains of the wild-type ER- α (Tora *et al.* 1989b, Tzukerman *et al.* 1994).

Similarly, overexpression of a single ER- α variant using stable transfection technology has resulted in different results in different laboratories (Fuqua & Wolf 1995, Rea & Parker 1996). Moreover, direct correlation of any single ER- α variant with clinical tamoxifen resistance or tamoxifen resistance of breast cancer cells in culture has not been forthcoming. Since most of these comparisons have been performed using individual ER- α variants and do not take into account the entire spectrum of ER- α variants relative to each other, the conclusions remain controversial. Together, the data support the hypothesis that the development of hormone independence and endocrine resistance in human breast cancer is a multifactorial process and indeed there are many examples where the development of estrogen-independent growth and antiestrogen resistance are dissociable events in breast cancer cell line models (Katzellenbogen *et al.* 1987, Welshons & Jordan 1987, Van der Berg *et al.* 1990, Brunner *et al.* 1993).

Similar to these and other studies, we have found that the development of estrogen-independent growth in a breast cancer cell line model, through long-term growth in estrogen-depleted medium, was not associated with antiestrogen resistance. Although the estrogen-nonresponsive T5-PRF cells have a reduced sensitivity to the pure antiestrogen ICI, their growth response to OT is identical to that of parental T5 cells (Coutts *et al.* 1996).

However, when we investigated the relative pattern of expression of ER- α -deleted variant mRNA in T5-PRF compared with parental T5 cells, there was a significant difference in the relative expression of a previously described exon 3/4-deleted ER- α variant mRNA (reviewed in Murphy *et al.* 1997). Although the question of whether this ER- α variant is a cause of estrogen independence or merely an effect of the selection process for estrogen independence requires further study, our data using transient transfection analyses tend to support a possible functional role for the putative 3/4-deleted ER- α protein encoded by the variant mRNA in the phenotype observed in T5-PRF human breast cancer cells.

In this study we have shown that T5-PRF cells have significantly increased ligand-independent (basal) ER- α activity (reflected in both ERE-tk-CAT activity and endogenous PR levels). The recombinant d3/4 variant ER- α was able to confer significantly increased ligand-independent (basal) and estrogen-responsive transcriptional activity when expressed in parental T5 cells and showed a trend towards increased basal transcriptional activity when coexpressed with wild-type ER- α in ER- α -negative human breast cell lines.

While the demonstrated effects of d3/4 on HEGO transcriptional activity in ER- α -negative cell lines suggest a putative functional role for this variant ER- α , this effect required equal or higher levels of d3/4 than HEGO. Although our data provide 'proof of principle' that the d3/4 variant can modulate the transcriptional activity of the wild-type ER- α the relevance of the expression levels of each protein achieved in the reconstituted transient expression system to the endogenous levels of ER- α and d3/4 variant expression in T5-PRF is unclear. Furthermore, differences in the background of transcriptional coactivators and corepressors between naturally ER- α positive and negative cell lines (for example Tzukerman *et al.* 1994), as well as the presence of other naturally occurring ER- α variants in naturally ER- α positive cell lines are all likely to impact on the final outcome of ER-mediated transcriptional activity and underlie the differences seen between the transiently manipulated cells and the naturally occurring T5-PRF phenotype. Moreover, expression of ER- β and/or its variants may influence estrogen action (Giguere *et al.* 1998). Both T5 and T5-PRF cells express low levels of ER- β mRNA determined by RT-PCR analysis (A S Coutts, H Dotzlaw, E Leygue & L C Murphy, our unpublished data); however, the functional significance of the levels remains unknown. Nonetheless, we saw a significant effect on ER- α ligand-independent transcription in T5 cells at levels of

cotransfected d3/4 that likely would not be higher than the endogenous ER- α in these cells, but the extrapolation of these data to the relative expression of wild-type ER- α and d3/4 variant in T5-PRF cells is presently unknown.

It is of significance that we can reproduce an effect of d3/4 variant in the parental T5 cells, which would likely contain a more representative background of ER- α accessory proteins (i.e. coactivators and/or corepressors) as well as other variant forms of ER- α , which would all contribute to the final ER-mediated biological response. As well, our data do not exclude the possibility that other alterations have occurred in T5-PRF cells which, in combination with an altered ER- α variant, may contribute to the estrogen-independent phenotype of T5-PRF cells (Coutts & Murphy 1998).

It has previously been shown that breast cancer cells can adapt to low levels of estrogen by enhancing their sensitivity to estrogen (Masamura *et al.* 1995). Estrogen deprivation of MCF-7 human breast cancer cells resulted in estrogen hypersensitivity and maximal growth was achieved with an estrogen concentration 4–5 orders of magnitude lower than wild-type cells. These researchers also found that the concentration of ICI needed to inhibit growth of these cells was ~ 6 orders of magnitude lower than wild-type cells, supporting the hypothesis in this model that increased sensitivity to ER ligands had occurred. While supersensitivity to estrogen in T5-PRF cells cannot be entirely ruled out, we have previously shown that in contrast to the data of Masamura *et al.* (1995), T5-PRF cells are less sensitive to growth inhibition by ICI, suggesting that in this model other mechanisms are likely involved.

Our data do not address the mechanism by which d3/4 enhances ER transcriptional activity, but several possibilities exist. The ER- α contains at least two separate regions that are required for optimal transcriptional activation (Tora *et al.* 1989b, Tzukerman *et al.* 1990). The N-terminal region contains promoter and cell-type-specific ligand-independent transcriptional activity (AF1) and the second, AF2, is located in the ligand-binding C-terminus of the receptor. d3/4 containing an intact AF2 or AF1 domain could interfere with, or sequester, an ER- α repressor protein resulting in increased ER- α transcriptional activity in the absence of ligand (Lee *et al.* 1996). This variant may also retain the ability to interact with other ER- α regulatory proteins such as coactivators or components of the basal transcription machinery.

ER- α also contains two domains involved in dimerization (Kumar & Chambon 1988, Schwabe *et al.* 1993). A weak dimerization interface is

present in the DBD and a strong interface is located in the C-terminal ligand-binding domain (White *et al.* 1991). d3/4 containing an intact C-terminal dimerization domain may form heterodimers with wild-type ER- α that have altered transcriptional regulatory properties through differing protein-protein interactions.

The crystal structure of the ER- α hormone-binding domain has recently been elucidated (Brzozowski *et al.* 1997). Based on this structure, the d3/4 protein would contain many of the regions essential for transactivation, including the predominant helix 12 (encompassing amino acids 539–547). However, since d3/4 alone has no transcriptional activity (at least on a classical ERE-regulated promoter) the structure must be sufficiently altered to prevent activity, or AF2 can only be activated in a ligand-dependent manner but d3/4 cannot bind ligand. Helix 12 in AF2 is believed to be the main region involved in coactivator recruitment and it may be possible that d3/4, following heterodimerization with ER- α , enhances recruitment of coactivators to the basal transcription complex and thus enhances ER- α activity.

We have found that the d3/4 caused increased ligand-independent wild-type ER- α activity and also enhances the ligand-induced ER- α transcriptional activity, despite the fact that on its own this variant is not transcriptionally active on a classical ERE promoter, nor does it bind ligand *in vitro* to any significant degree. Studies have demonstrated that the ability of steroid hormone receptors to modulate transcription does not necessarily require that the receptors bind DNA. PRC, an N-terminally truncated PR isoform lacking the first zinc-finger of the DBD, has no transcriptional activity of its own but has been shown to enhance progestin-induced transcriptional activity (Wei *et al.* 1996). The DBD of the ER- α does not appear to be necessary for raloxifene activation of the transforming growth factor (TGF)- β gene (Yang *et al.* 1996) and ER- α can activate transcription from AP-1 dependent promoters through a DNA-binding-independent pathway (Webb *et al.* 1995). Sp1 and ER- α directly interact to enhance estrogen-induced transactivation of the Sp1-dependent Hsp27 gene promoter and the DBD of the ER- α is not required (Porter *et al.* 1997).

Recent research has demonstrated that the ER- α can be activated in a ligand-independent fashion (Ignar-Trowbridge *et al.* 1993). Studies have shown that several growth factors such as EGF, TGF- α and insulin-like growth factor-I were able to activate the ER- α in the absence of ligand. The ability to activate the ER- α in the absence of estrogen could confer a growth advantage to breast

cancer cells and aid in the development of a hormone-independent phenotype. The presence of alternate forms of ER- α capable of interacting with wild-type ER- α to increase ligand-independent activity could also confer a potential growth advantage to breast cancer cells. A recent study has shown that constitutively active, ligand-independent ER- α mutants undergo conformational changes and interactions with coactivators that mimic changes in ER- α that are usually regulated by ligand (Lazennec *et al.* 1997). Recently, researchers have shown that thyroid hormone receptor- β 2 (TR- β 2) is a ligand-independent activator of the gene encoding thyrotropin-releasing hormone and have mapped a region in the N-terminus of the receptor responsible for this activity (Langlois *et al.* 1997). These researchers suggest that the mechanism of ligand-independent activation involves direct interaction of the TR- β 2 N-terminus with either transcriptional cofactors or the basal transcription machinery itself.

An increased relative expression of variant ER- α proteins containing intact AF domains, could result in increased interactions with the ER- α and/or other proteins involved in ER- α transcriptional activity. This could be a potential mechanism for estrogen-independent growth associated with the presence of one or more variant ER species and could explain the increased ER- α activity we have seen with the d3/4.

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