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Characterization of Stably Transfected Fusion Protein GFP-Estrogen Receptor-α in MCF-7 Human Breast Cancer Cells

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Abstract Tagging hormone receptors with the green fluorescent protein (GFP) has increased our knowledge of ligand dependent sub-cellular trafficking of hormone receptors. However, the effect of the tagged hormone receptor expression on the corresponding wild type hormone receptor and endogenous gene expression has not been investigated. In this study, we constructed a MCF-7 cell line stably expressing GFP-tagged human estrogen receptor-α (ER) under control of the tetracycline-on system to determine the effect of GFP-ER expression on cell proliferation and expression of endogenous ER and hormone-responsive genes. Further, the inducible system was applied to determine the ligand dependent turnover rates of GFP-ER protein and mRNA. Our results demonstrate that GFP-ER expression did not affect cell cycling. Independent of ligand, GFP-ER markedly reduced the level of endogenous ER mRNA and protein, suggesting that ER negatively autoregulates its expression. Cisplatin cross-linking studies showed that GFP-ER is associated with nuclear DNA in situ, suggesting that GFP-ER is partially replacing ER at estrogen response elements. Furthermore, GFP-ER expression did not affect the estradiol induced temporal expression of hormone responsive genes c-*myc* and pS2. J. Cell. Biochem. 86: 365–375, 2002.

Key words: estrogen receptor; green fluorescent protein; nuclear matrix; subcellular localization; cisplatin cross-linking; protein half-life

The human estrogen receptor-α (ER) belongs to the steroid nuclear receptor family and is expressed in high levels in approximately 77% of breast cancers. The presence of ER in tumors correlates with estrogen dependence in breast cancer. Cell proliferation of hormone-dependent breast cancer cells increases when estrogen/17-β estradiol (E_2) binds to ER, which also induces a decline in ER mRNA and protein levels [Saceda et al., 1988; Borras et al., 1994]. Ligands that compete with E_2 for ER binding, such as the partial anti-estrogen, 4-hydroxytamoxifen (OH-TAM), and the pure anti-estrogen, ICI 182,780, decrease cell proliferation and alter ER levels [Robertson, 1996]. These ligands also affect the nuclear localization of ER. ER is located in the nucleus and exists in two distinct forms, either a loosely- or tightly-bound to the three-dimensional RNA-protein network of the nuclear matrix. When bound to ligand, ER is tightly bound to the nuclear matrix and has a speckled nuclear location [Press et al., 1989; Htun et al., 1999; Stenoien et al., 2000, 2001].

Fluorescently tagged proteins are informative biological tools for studying the subcellular trafficking of hormone receptors [Hager et al., 2000]. Previously, we analyzed

Abbreviations: GFP, green fluorescent protein; ER, estrogen receptor α; E2, 17-β estradiol; OH-TAM, 4-hydroxytamoxifen; ERE, estrogen responsive element; rtTA, reverse tetracycline; FBS, fetal bovine serum; PRF-DMEM, phenol red free Dulbecco's Modified Eagle Medium; DOX, doxycycline; NM-IF, nuclear matrix with attached intermediate filaments.

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the sub-cellular location of transiently expressed green fluorescent protein (GFP)-tagged ER in breast cancer cell lines. The GFP-ER was functional in that the fusion protein activated an estrogen responsive element (ERE)-containing reporter gene in the presence of E_2, while ICI 182,780 inhibited this activity. GFP-ER showed the same ligand dependent sub-cellular localization patterns in the nucleus as wild type ER [Htun et al., 1999].

The effect of the tagged ER expression on the corresponding endogenous ER and hormone responsive gene expression has not been investigated. To control the expression of the GFP-ER transgene in an ER positive hormone dependent breast cancer cells, we isolated a MCF-7 cell line that stably expresses GFP-ER under control of the doxycycline-on system [Gossen et al., 1995]. Besides providing information on the effect of GFP-ER expression on cell cycling and endogenous gene expression, this inducible system allowed us to determine the effects of ligands on GFP-ER mRNA and protein turnover rates in human breast cancer cells.

MATERIALS AND METHODS

Construction of Stable Cell lines Expressing GFP-ER

The pUHD172-1neo plasmid that includes the sequence for the reverse tetracycline repressor (rtTA) fused to the activating domain of VP16 protein and a neomycin resistance gene was stably transfected into the MCF-7 human breast cancer epithelial cell line, yielding clone 89 (gift from Dr. R.P.C. Shiu). Clone 11, a stable cell line expressing GFP-ER, was created through transfection of a second plasmid, pUHD10-3, which contains N-terminally tagged GFP-ER under the control of a tetracycline inducible promoter, into clone 89. In the absence of doxycycline (DOX), a tetracycline analog, rtTA, does not activate the expression of GFP-ER, but when DOX is added the rtTA-VP16 fusion protein can bind to tetracycline responsive elements upstream of the GFP-ER, resulting in the expression of GFP-ER.

Cell Culture and Treatments

Clone 89 and 11 were maintained in 600-ml angle-necked flasks (Nunc, Burlington, Ontario) containing Dulbecco's Modified Eagle Medium supplemented with 0.37% (w/v) $NaHCO_3$, 7% (v/v) Fetal Bovine Serum (FBS), 2.2 mM L-glutamine, 0.33% (w/v) glucose, Penicillin/Streptomycin (100 U/ml/100 μg/ml), 0.1 mM non-essential amino acids, and 0.2 mg/ml Hygromycine B. Geneticin (0.2 mg/ml) was added to clone 11 cells only (all reagents from GIBCO/BRL, Burlington, Ontario). Environmental conditions were maintained at 37°C with humidified atmosphere, 5% CO_2/95% air.

Cells were reseeded in phenol red free Dulbecco's Modified Eagle Medium (PRF-DMEM-Sigma-Aldrich, Oakville, Ontario) and supplemented with 0.37% (w/v) $NaHCO_3$, 7% (v/v) 2× charcoal stripped FBS, 2.2 mM L-glutamine, 0.33% (w/v) glucose, penicillin/streptomycin (100 U/ml/100μg/ml), and 0.1 mM non-essential amino acids (GIBCO). Doxycycline (0.1, 0.5, and 1.0 μg/ml) were also present in the medium. Ten nM final concentration of E_2 (Sigma-Aldrich), OH-TAM (Research Biochemicals International, Natick, MA), or ICI 182,780 (ICI) (TOCRIS, Ballwin, MO) was added at indicated time points before harvesting. For harvesting, cells were either lyzed on tissue culture plates for protein and RNA analyses or trypsinized and collected for fluorescent-activated cell sorting [Chadee et al., 1995], nuclei preparation, or nuclear matrix preparation [Samuel et al., 1997]. Bivariant analysis of GFP-ER and cell cycle was done as described [Rasko, 2001].

GFP-ER mRNA and Protein Turnover Studies

Cells were seeded in PRF-DMEM with the additions noted above as well as 1.0 μg/ml of DOX (Sigma-Aldrich). The cells were allowed to grow for 4–6 days prior to treatment. Ten nM E_2, OH-TAM, ICI, or ethanol vehicle was added to the plates for each time point. After 6, 18, 20, or 22 h, DOX was removed and the cells were allowed to grow until they had been exposed to ligand for a total of 24 h. In addition, plates that were exposed to ligands for 24 h without removal of DOX were prepared. Cell numbers were determined by FACS using a Z2 Coulter Counter. For protein analysis, cells were resuspended in 4× SDS–PAGE sample buffer containing 1 mM PMSF but no dye to a concentration of 5,000 cells per microliter. The lysates were stored at −20°C. Cells used for RNA analysis were harvested in RLT buffer (RNeasy kit from Qiagen, Mississauga, Ontario). The RNA was isolated according to the Animal

Cell Protocol from the RNeasy kit and stored at −70°C.

Cell Fractionation

Nuclear and cytosolic fractions were prepared as previously described [Samuel et al., 1997]. Briefly, cells ($3–4 \times 10^7$) were suspended in TNM buffer (300 mM sucrose, 100 mM NaCl, 2 mM $MgCl_2$, 10 mM Tris pH 8.0, and 1% thiodiglycol) with 0.5% Triton X-100, 1 mM PMSF, and EDTA-free protease inhibitor cocktail tablets (one tablet per 50 ml Boehringer Mannheim). The cells were homogenized using a Dounce homoginizer five times and passed through an 18-gauge needle three times. The suspension was centrifuged, and the supernatant containing the cytosol and proteins loosely bound in the nucleus was collected. The nuclei in the pellet fraction were re-suspended in TNM buffer and collected by centrifugation. This fraction contains proteins tightly bound in the nucleus [Sun et al., 2001]. Nuclear matrices were prepared as described previously [Samuel et al., 1998]. Briefly, nuclei digested with DNase I were extracted with 0.25 M ammonium sulfate, centrifuged, and the pellet (nuclear matrix with attached intermediate filaments, NM-IF) was collected.

Immunoblot Analyses

Immunoblot analyses were performed as described previously, with anti-ER antibodies (6F11, Novacastra, Newcastle upon Tyne, UK) [Sun et al., 2001]. Detection was accomplished using the ECL system according to the manufacturer's instructions (Amersham Pharmacia Biotech, Arlington Heights, IL). To standardize the net intensity values of the blots, we employed a standard curve of known values and net intensities. To generate the standard curve, increasing volumes of a known concentration were run on an SDS–PAGE gel and immunoblotted as described previously. The blots were scanned into the Image Station 440CF (Kodak Digital Science™) and the net intensity of the GFP-ER or ER bands were determined using 1D Image Analysis Software (Kodak Digital Science™). The net intensity of each band was plotted against the volume loaded and the best linear trendline was determined (Microsoft Excel). The equation for the trendline was used to calculate the relative loading volumes from the net intensity reading for each experimental sample, which were used for subsequent graphs. For turnover data, the relative loading volumes were used to create a graph and the linear regression lines for each curve were determined (SigmaPlot). The regression lines yielded data points that were used to calculate the half-life of GFP-ER ($t½ = 0.693k$, where $k = \ln[n1/n2]/[t2-t1]$).

Northern Blot Analyses

Ten micrograms of total RNA was loaded onto the 1.25% formaldehyde agarose gel and was transferred to nylon transfer membrane (Magna Graph, 0.45×10^{-4} m). The blot membrane was hybridized with ^{32}P labeled cDNA probes (2×10^6 cpm/ml). The membranes were exposed to X-ray film or to Kodak Storage Phosphor Screens (Bio-Rad, Hercules, CA), which were then scanned into the Personal Molecular Imager® FX (Bio-Rad). From the scans, the net intensities of the GFP-ER or ER mRNA bands were determined using Quantity One software (Bio-Rad). The net intensities of the GFP-ER mRNA bands were used to create a graph and the linear regressions for each curve were determined. The half-life of the GFP-ER mRNA was then calculated as for protein.

Cisplatin Cross-Linking

Cisplatin cross-linking of breast cancer cells was done as described previously [Holth et al., 1998].

RESULTS

Induction of GFP-ER in Clone 11, a Stably Transfected MCF-7 Cell Line

The responsiveness of the tetracycline (DOX)-on system in expressing GFP-ER in clone 11 MCF-7 cells was determined in immunoblot experiments. In the absence of DOX, GFP-ER was not detected. Figure 1 shows that there was a progressive increase in GFP-ER levels as the DOX concentration was elevated, with the levels of GFP-ER approaching a maximum after 0.5 μg/ml DOX. In estrogen-replete conditions, the levels of GFP-ER attained at the various DOX concentrations were lower than those in cells grown under estrogen deplete conditions.

The GFP-ER content in cell cycle sorted cells was determined by sorting propidium iodide-stained GFP-ER-expressing cells by FACS. Under estrogen deplete or replete conditions, GFP-ER was expressed at similar levels in G1, S, and G2/M cells (Fig. 2). The results also show

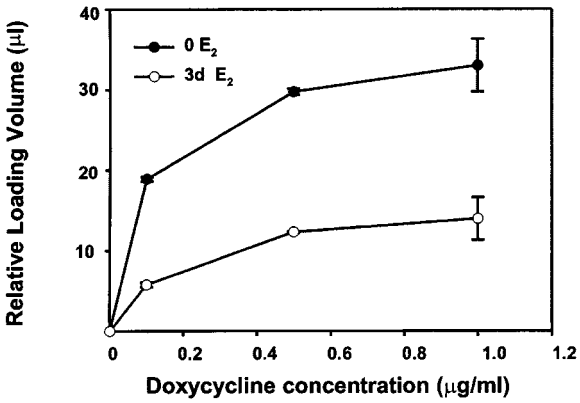

Fig. 1. GFP–ER protein levels in MCF-7 human breast cancer cells. Clone 11 cells were cultured in PRF–DMEM containing 7% 2 × CS–FBS. Different concentrations of DOX were added to induce GFP–ER expression. Estradiol (10 nM) was added for 3 days before harvesting. Whole cell lysates from equal number of cells were resolved by 10% PAGE followed by immunoblot analyses with a mouse anti-ER antibody. The results from two experiments are shown.

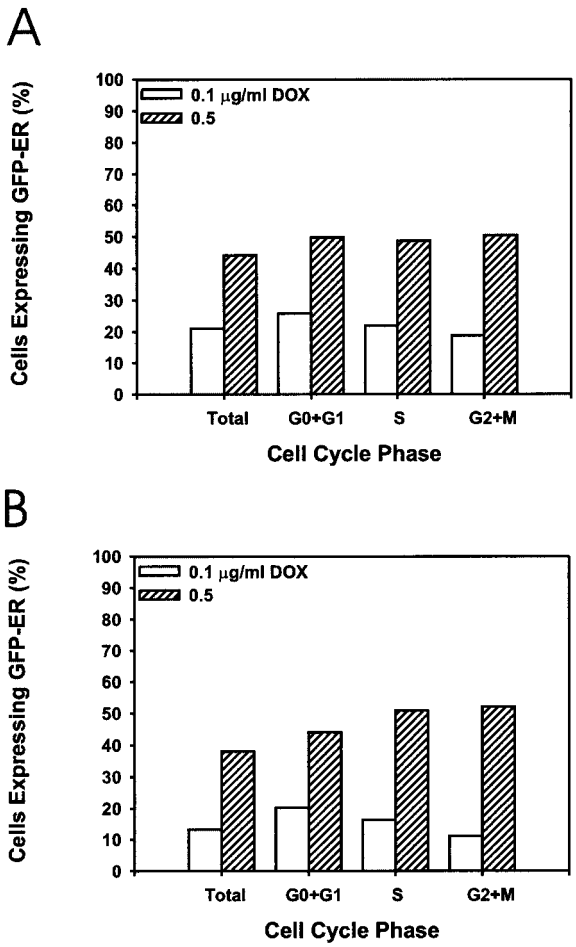

Fig. 2. GFP–ER expression in cell cycle sorted cells. Clone 11 cells were cultured in PRF–DMEM containing 7% 2 × CS–FBS with increasing concentrations of DOX. Ten nM of E_2 was added for 0 (**A**) or 3 days (**B**) prior to harvest. Cells were fixed in 70% ethanol, then digested with DNase-free RNase A, and stained with propidium iodide before analysis by FACS. The figure shows a representative results of an experiment repeated twice.

that elevating the DOX concentration from 0.1 to 0.5 μg/ml increased the number of cells expressing GFP-ER. Further, cells cultured under estradiol replete and deplete conditions responded similarly to DOX. Thus, the decreased level of GFP-ER in cells cultured under estradiol replete (Fig. 1) was due to a decrease in GFP-ER expression per cell rather than a decreased number of cells expressing GFP-ER.

Effect of DOX Induction of GFP-ER on Growth and Cell Cycle Distribution

Previous studies demonstrated that expression of an ER transgene in ER positive breast cancer cells did not adversely affect E_2-stimulated proliferation of these cells [Zajchowski et al., 1993; Lazennec et al., 1999]. To determine whether GFP-ER expression and/or DOX had an effect on cell growth and cycling, the doubling time was calculated for clone 89 and 11, and the percentage of cells in S + G2/M phases was determined by FACS. To determine doubling times, cells were grown in different concentrations of DOX in the presence of 10-nM E_2 and counted daily for 7 days using a Coulter counter. The calculated doubling times for clone 89, which do not express GFP-ER, suggest that DOX had no effect on cell growth as evidenced by doubling times of 1.63 ± 0.17 days ($n = 3$) and 1.71 ± 0.19 days ($n = 3$) for cells treated with no and 1.0 μg/ml DOX, respectively. Expression of GFP-ER did not alter the doubling time of the cells which, at 1.59 ± 0.08 days ($n = 3$) with 1.0 μg/ml DOX, is comparable to the control clone 89 values. FACS analysis was then used to determine the effect of DOX and GFP-ER expression on cell cycling. Figure 3 shows the percentage of cells (clone 89 and $11 \pm$ DOX and $\pm$ 10 nM E_2 for 3 days) in S + G2 + M phases of the cell cycle. Under estradiol deplete conditions, the cell cycle distribution of clone 89 cells was not affected by increased concentrations of DOX (Fig. 3A). Addition of 10-nM E_2 for 3 days resulted in an increase of cells in S + G2/M phases and DOX addition had no effect (Fig. 3B). Clone 11 cells displayed similar results, demonstrating that increased GFP-ER expression

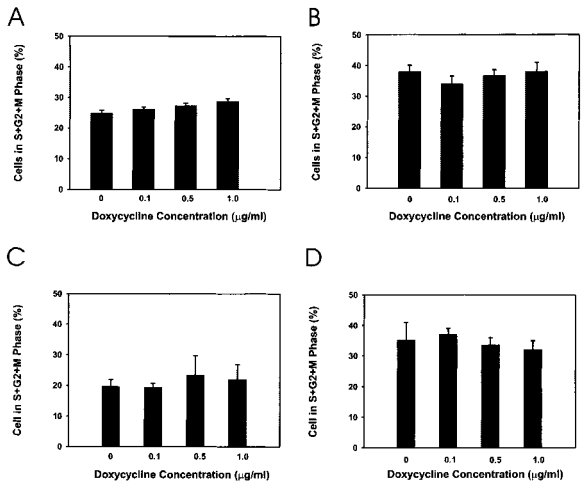

Fig. 3. Effect of GFP–ER expression on cell cycling. MCF-7 clone 89 and 11 cells were cultured in PRF-DMEM containing 7% 2 × CS–FBS with increasing concentrations of DOX. Ten nM of E_2 was added for 0 or 3 days prior to harvest. Cells were fixed in 70% ethanol, then digested with DNase-free RNase A, and stained with propidium iodide before analysis by FACS. **A**: Addition of 10-nM E_2 for 3 days results in an increase of cells into S + G2/M phase, suggesting that more cells are actively cycling (**B**). Clone 11 cells display similar results, demonstrating that increased GFP-ER levels do not affect cell cycling (**C,D**). The results from three experiments are shown.

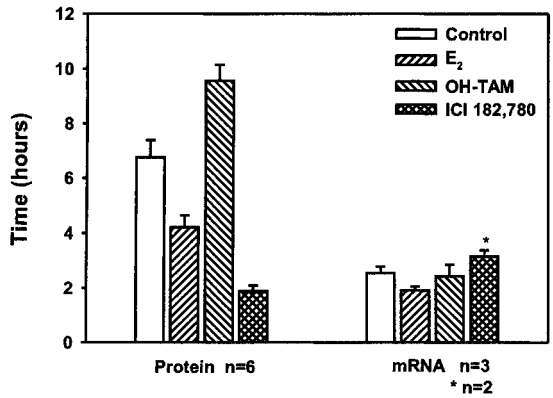

Fig. 4. Half-life of GFP-ER protein and mRNA. Clone 11 cells were incubated for 4–6 days in PRF–DMEM containing 7% 2 × CS–FBS and 1.0 μg/ml DOX. Medium was then removed and replaced by fresh medium in the absence (control) or presence of specific ligands (10 nM). After 6, 18, 20, or 22 h post-addition of ligand, the medium was changed to PRF–DMEM with ligand, lacking DOX. Incubation continued until 24-h post-addition of ligand. Cells were used for immunoblot and Northern blot analysis and scanned. Densitometric analysis was used to determine half-life ($t½ = 0.693k$, where $k = \ln[n1/n2]/[t2-t1]$). The results from six experiments (protein analyses) and two or three experiments (mRNA analyses) are shown.

under estrogen deplete and replete conditions did not affect cell cycling (Fig. 3C,E). Our results demonstrated that DOX and GFP-ER expression did not affect cell growth, cell cycling, or cell cycle distribution of the breast cancer cells.

Turnover Rates for GFP-ER Protein and mRNA

The reduced levels of GFP-ER expressed in clone 11 cells cultured under estradiol replete conditions suggested that GFP-ER protein and/or mRNA turnover was increased. Previous studies have demonstrated that the half-life of ER protein is ligand dependent [Eckert et al., 1984; Alarid et al., 1999; Nawaz et al., 1999; Lonard et al., 2000]. To determine the half-life of GFP-ER protein and mRNA, clone 11 cells were first treated with DOX and 10 nM ligand (E_2, OH-TAM or ICI), then the DOX was removed at successive time points and the samples prepared for immunoblot or Northern blot analyses. Immunoblot analyses of GFP-ER levels showed that the removal of DOX from the growth media resulted in a decrease in GFP-ER protein levels, with the rate of GFP-ER degradation being ligand dependent. GFP-ER turnover was greater in E_2-treated cells compared to cells cultured under estradiol deplete conditions. GFP-ER turnover was greatest in cells cultured with ICI and slowest in cells incubated with OH-TAM. Densitometric analyses revealed that the half-life of the unoccupied GFP-ER was 6.7 (± 0.64) h (Fig. 4). Both E_2- and ICI-bound receptor had shorter half-lives of 4.2 (± 0.44) and 1.9 (± 0.20) h, respectively (37 and 78% shorter). OH-TAM-bound receptor had a half-life of 9.6 (± 0.58) h, which was 43% longer than for unoccupied receptor. The removal of DOX from the growth media resulted in a decline in GFP-ER mRNA levels at similar rates for all treatment groups (Fig. 4). Therefore, the half-life for GFP-ER mRNA was independent of ligand. Thus, the differential levels of GFP-ER protein expressed in clone cells cultured under estradiol deplete and replete conditions was a consequence of differential turnover rates.

Cellular Distribution of GFP-ER and Endogenous ER

When ligand (E_2, OH-TAM, or ICI) is added to hormone dependent breast cancer cells, there is a rapid recruitment of ER to the nuclear matrix, and the ER goes from a loosely bound to a tightly bound nuclear state [Htun et al., 1999; Stenoien et al., 2000, 2001; Sun et al., 2001]. In this study, clone 11 cells cultured under estradiol deplete conditions was incubated for 5 h with 10 nM of

E_2, OH-TAM, or ICI. Whole cell extracts and fractions containing loosely-bound and tightly-bound nuclear proteins were analyzed by immunoblotting to elucidate the cellular distribution of ER and GFP-ER. Figure 5 (cell lysate fractions) shows that steady state of GFP-ER and ER varied with ligand, with the level of GFP-ER and ER decreasing in the order of OH-TAM > no ligand > E_2 > ICI. These levels of GFP-ER and ER protein reflected the turnover rates for GFP-ER. In the absence of ligand, GFP-ER and ER partitioned principally in the loosely-bound nuclear fraction (i.e., nuclear proteins that readily leave nuclei treated with Trition X-100). Five hours following the addition of ligand, there was a shift in the partitioning of GFP-ER and ER from the loosely bound nuclear form to the tightly bound nuclear form. The results show that GFP-ER and ER ligand dependent subcellular trafficking occurred in parallel.

To confirm that GFP-ER was being recruited to the nuclear matrix, we monitored the association of GFP-ER with the nuclear matrix as a function of time following the addition of 10-nM E_2 to clone 11 cells cultured under estradiol deplete conditions. Figures 6 shows that an increased association of GFP-ER was observed at 30 min, and that the recruitment of GFP-ER to the nuclear matrix increased over 4 h. Similarly, the addition of OH-TAM and ICI for 4 h resulted in the increased association of GFP-ER with the nuclear matrix.

Cisplatin Cross-Linking Studies

Cisplatin preferentially cross-links nuclear matrix proteins to DNA [Samuel et al., 1998; Spencer et al., 2001]. We have demonstrated previously that cisplatin will cross-link ER to nuclear DNA in situ in human breast cancer cells. We investigated whether GFP-ER and ER were associated with nuclear DNA before addition of E_2 and whether there was a change in the binding of GFP-ER and ER following 30 min of E_2 addition. Figure 7 shows that the GFP-ER and ER were cross-linked to DNA in situ with cisplatin; however, the extent of cross-linking for both proteins increased after E_2 addition. Histone deacetylase 1, which is associated with the nuclear matrix and is cross-linked to DNA with cisplatin [Samuel et al., 1998], served as a loading control. The results show that E_2 enhanced the binding of ER and GFP-ER to ER DNA binding sites (EREs).

Effect of GFP-ER Expression on Endogenous ER Levels

During our immunoblot analyses of DOX induction of GFP-ER in clone 11 cells, we observed that endogenous levels of ER decreased as GFP-ER levels increased (Fig. 8A,B). Control studies with clone 89 showed that DOX at 0.1, 0.5, and 1.0 μg/ml did not alter the endogenous ER (data not shown). Northern blot analyses were done to determine the effect of GFP-ER expression on endogenous ER mRNA levels. Clone 11 cells cultured under estradiol deplete conditions were incubated with 0, 0.1, 0.5, or 1.0 μg/ml DOX and with 10-nM E_2 for 8 or 24 h. Figure 8C,D show that GFP-ER mRNA levels increased as a function of elevated DOX concentrations. This DOX-dependent increase in GFP-ER mRNA levels was not affected by the addition of E_2 to clone 11 cells (Fig. 8D). In

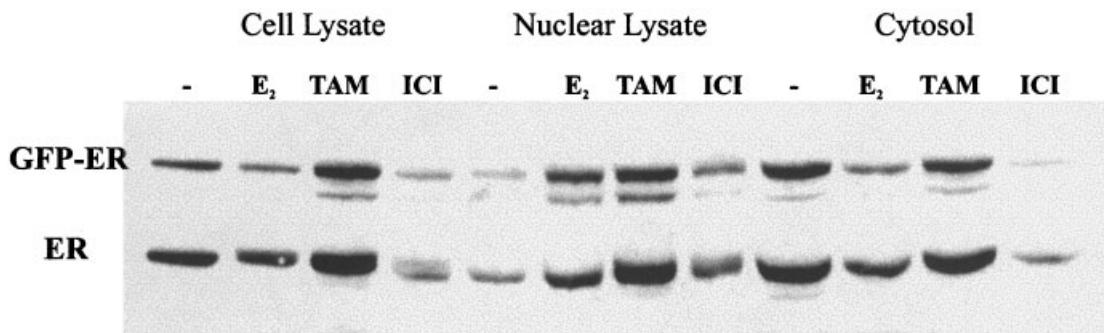

Fig. 5. Cellular distribution of GFP–ER and endogenous ER. Clone 11 cells were cultured in PRF–DMEM containing 7% 2 × CS–FBS and 1.0 μg/ml DOX. Five hours after addition of 10-nM ligand (E_2, estradiol; TAM, hydroxytamoxifen; ICI 182,780), the cells were harvested. Cells were separated into nuclear and cytosolic fractions and run on an SDS–PAGE gel along with cell lysates and immunoblotted as described in the legend to Figure 1. The figure shows a representative results of an experiment repeated twice.

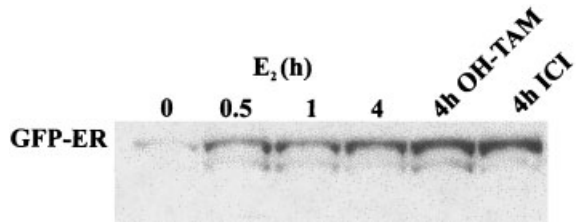

Fig. 6. Association of GFP–ER with the nuclear matrix. Clone 11 cells were cultured in PRF–DMEM containing 7% 2 × CS–FBS and 1.0 μg/ml DOX. The cells were harvested at various time points after the addition of 10-nM E_2 or 4 h after addition of OH-TAM or ICI. The nuclear matrix fraction (NM1–IF) was isolated. The nuclear matrix proteins were resolved by SDS–PAGE, immunoblotted as described in the legend to Figure 1. The figure shows a representative results of an experiment repeated three times.

contrast, Figure 8C,E show that the steady state levels of ER mRNA declined following the addition of E_2. Further, Panel C and E show that in the absence or presence of E_2, increased GFP-ER expression decreased ER mRNA levels. These results suggest that GFP-ER is negatively regulating the expression of the endogenous ER gene. Further together with our results with cisplatin cross-linking, our data suggest that GFP-ER is binding to or is replacing ER at EREs.

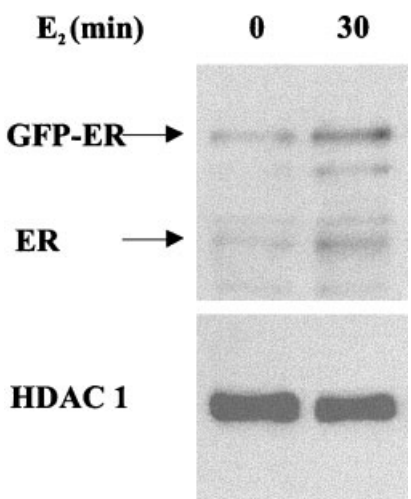

Fig. 7. Cisplatin cross-linking of GFP–ER to DNA. Clone 11 cells were cultured in PRF–DMEM containing 7% 2 × CS–FBS and 1.0 μg/ml DOX. Ten nM of E_2 was added for 0 or 30 min prior to cisplatin cross-linking. The samples were resolved by SDS–PAGE and immunoblotted as described in the legend to Figure 1. The figure shows a representative results of an experiment repeated twice.

Effect of GFP-ER on Expression of Endogenous Hormone-Responsive Genes

To determine the effect of GFP-ER expression on the induction of estrogen responsive genes (c-*myc* and pS2), Northern blot analysis was performed with mRNA isolated from clone 11 cells cultured in 0 or 1.0 μg/ml DOX under estradiol deplete conditions and incubated for various times with 10-nM E_2. Figure 9A shows that c-*myc* mRNA levels increased, reaching a maximum at 1 h of E_2 exposure, and then declined. GFP-ER expression did not interfere with this process. pS2 mRNA levels increased during the entire time course for cells with and without GFP-ER expression (Fig. 9B). These results show that GFP-ER does not interfere with the induction of estrogen responsive genes.

DISCUSSION

Others and we analyzed ligand dependent ER subcellular trafficking by monitoring GFP-ER location [Htun et al., 1999; Stenoien et al., 2000, 2001]. In this study, we determined the effects of estrogen on cell cycling of GFP-ER expressing cells and on GFP-ER mRNA and protein turnover rates. The DOX-regulated GFP-ER expression system was not leaky and graded expression of GFP-ER was observed in response to increasing DOX concentrations. Bivariant FACS and immunoblot analyses demonstrated that raising the DOX levels increased the number of MCF-7 clone 11 cells expressing GFP-ER. These observations suggested that raising the DOX concentration increased the probability of the reverse Tet repressor-VP16 fusion proteins binding to heptomerized tet-operators and activating the minimal CMV promoter [Gossen and Bujard, 1992; Baron and Bujard, 2000; Fiering et al., 2000].

In accordance with studies using hormone-dependent breast cancer cells, expression of the GFP-ER transgene in MCF-7 human breast cancer cells did not affect E_2-stimulated cell cycling. Further, we did not observe GFP-ER expressing cells undergoing apoptosis, indicating that GFP was not toxic to the cells [Liu et al., 1999]. In contrast, E_2 inhibits the proliferation of hormone-independent breast cancer cells and nontumorigenic breast epithelial cells expressing an ER transgene [Jiang and Jordan, 1992; Zajchowski et al., 1993; Levenson and Jordan, 1994; Lundholt et al., 1996; Pilat et al., 1996; Wang et al., 1997]. Evidence suggests that

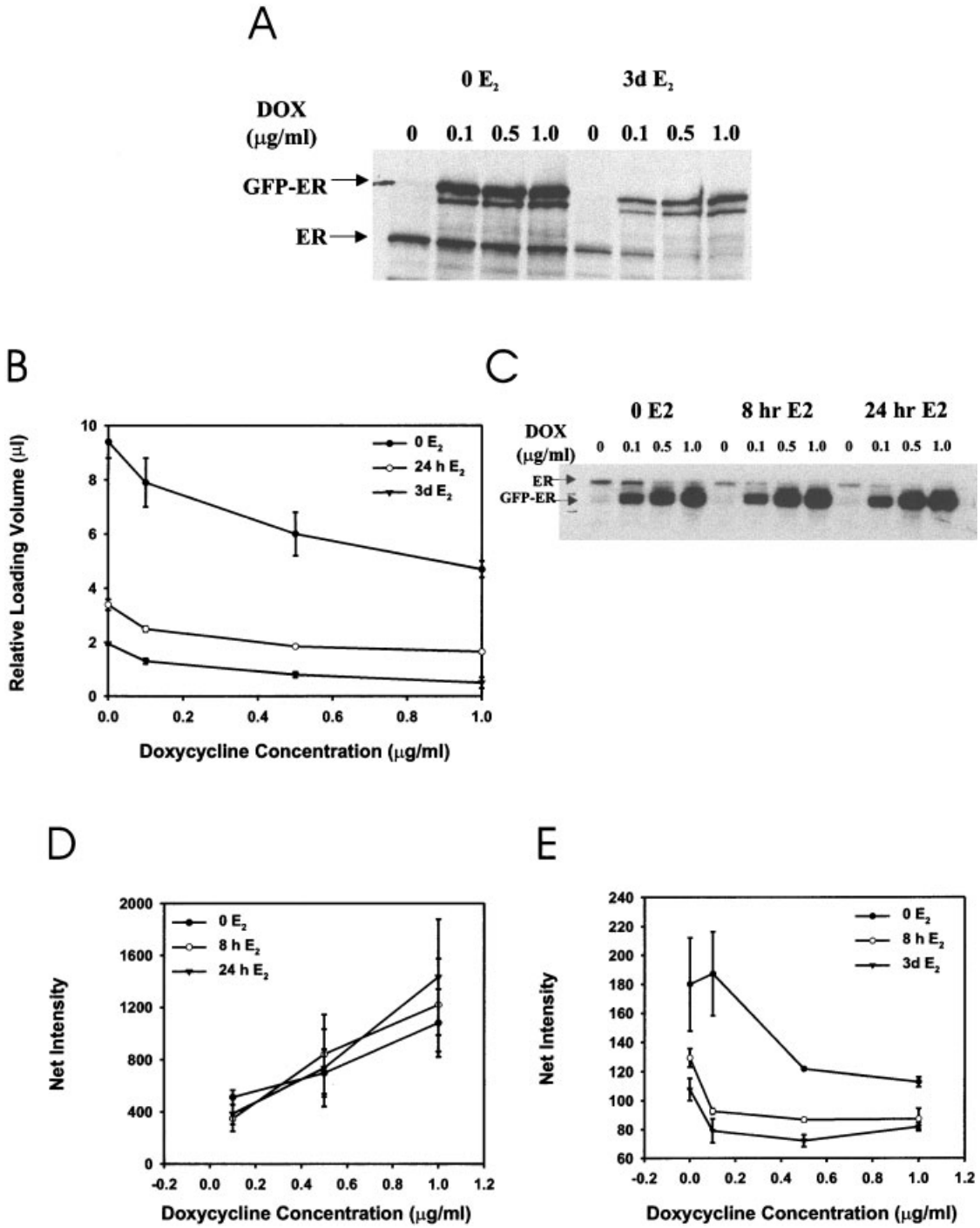

Fig. 8. Effect of GFP–ER expression on endogenous ER. **A**: Clone 11 cells were cultured in PRF–DMEM containing 7% 2 × CS–FBS. Increasing concentrations of DOX was added to induce GFP–ER expression. Following 3 days with 10-nM E_2, cells were harvested. Proteins in whole cell lysates from equivalent cell numbers were resolved by 10% SDS–PAGE and immunoblotted as described in the legend to Figure 1. **B–E**: Clone 89 (control) and clone 11 cells were cultured in PRF–DMEM containing 7% 2 × CS–FBS with increasing concentrations of DOX. Ten nanomoles of E_2 was added and the cells harvested at various time points, thereafter. The cells were used for either immunoblot (B) or Northern analysis (C–E). B: Data from imaging the immunoblot stained with anti-ER antibody were plotted versus concentration of DOX (n = 2). C: Northern blot showing expression of GFP–ER and ER mRNA at various concentrations of DOX and with or without E_2 for 8 or 24 h. D: Data from phosphoimager of Northern blots (GFP–ER mRNA) were plotted against concentration of DOX (n = 2). E: Data from phosphoimager of Northern blots (ER mRNA) were plotted against concentration of DOX (n = 2).

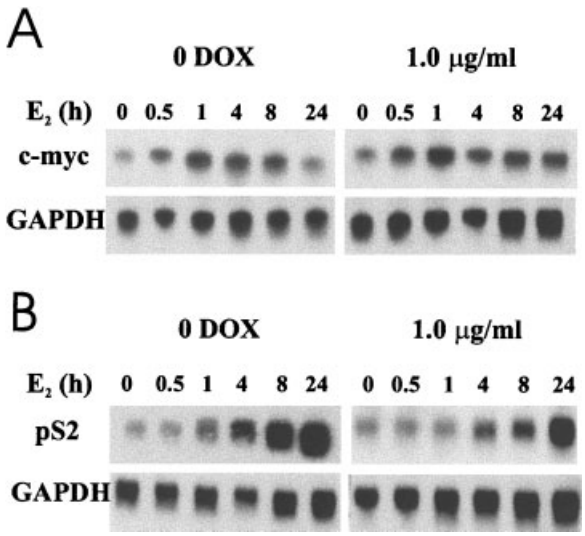

Fig. 9. GFP–ER and expression of endogenous genes. Clone 11 cells were cultured in PRF–DMEM containing 7% 2 × CS–FBS with 0 or 1.0 μg/ml DOX. Ten nM E_2 was added and the cells harvested at various time points, thereafter. The RNA was isolated, run on a formaldehyde agarose, and Northern blotted. The blots were hybridized with radiolabeled c-*myc*, pS2, and GAPDH cDNA and exposed to film. The figure shows a representative results of an experiment repeated three times.

E_2-ER in hormone-independent breast cancer cells increases the expression of the Cdk2 inhibitor, p21 (CIP1/WAF1) preventing entry of the cells into S phase of the cell cycle [Wang et al., 1997; Thomas et al., 1998].

Independent of E_2 and in response to increasing concentrations of DOX, there was a proportional increase in GFP-ER mRNA. In contrast, ER mRNA half-life decreases when E_2 is added Saceda et al., 1988, 1989, 1998]. However, similar to endogenous ER, GFP-ER protein stability was ligand dependent [Eckert et al., 1984; Saceda et al., 1988; Dauvois et al., 1992; Borras et al., 1996]. E_2 and pure anti-estrogens (ICI 182,780) decreased the half-life of GFP-ER, while partial anti-estrogens increased the half-life of GFP-ER [Borras et al., 1996]. To date, a single comprehensive analysis has not been undertaken regarding the calculation of the half-life of ER protein, when ER is in its unoccupied state or bound to the ligands E_2, OH-TAM and ICI 182,780. Previous studies have focused on a different selection of ligands, or did not calculate the actual half-life, mentioning instead the changes in the steady state levels of ER protein [Eckert et al., 1984; Dauvois et al., 1992; Borras et al., 1996; Pink and Jordan, 1996; Wijayaratne and McDonnell, 2001]. Our ligand set represents an estrogen (E_2), a partial anti-estrogen used in breast cancer treatment and prevention (OH-TAM) and a pure anti-estrogen used in clinical trials (ICI 182,780, also known as Faslodex).

The 26S proteasome has been implicated in the degradation of human ER in various cell lines including MCF-7 [Alarid et al., 1999; Nawaz et al., 1999; Wijayaratne and McDonnell, 2001]. It has been suggested that ER turnover contributes to ER's transcriptional activity [Lonard et al., 2000]. E_2 induction of estrogen-responsive genes (pS2) was hindered in MCF-7 cells incubated with the proteasome inhibitor MG132. However, our data show that E_2 induced expression of estrogen responsive genes (c-*myc* and pS2) is not diminished in MCF-7 cells, expressing high levels of GFP-ER. We conclude that E_2 enhanced turnover of ER is not a factor in modulating the transcriptional activity of ER.

GFP-ER is a functional ligand-activated transcription factor [Htun et al., 1999]. Cisplatin cross-linking studies show that GFP-ER is bound to nuclear DNA in cells grown under estrogen deplete and replete conditions. The addition of E_2 to breast cancer cells increases the binding of GFP-ER to nuclear DNA and to the nuclear matrix. Previous reports provided evidence for several ER binding sites in the ER promoter. Further, these studies demonstrated that ER autoregulates its expression, with estradiol enhancing ER promoter activity [Castles et al., 1997; Treilleux et al., 1997]. Our studies found that independent of E_2 GFP-ER expression decreases the expression of ER, providing evidence that ER negatively autoregulates its expression. The cisplatin cross-linking and immunoblot studies suggest that GFP-ER partially replaces ER at EREs. These observations suggest that GFP-ER may substitute for ER in the pS2 and c-*myc* promoter, without compromising temporal ligand dependent activation of these genes.

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