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The Human Estrogen Receptor-α Isoform hERα46 Antagonizes the Proliferative Influence of hERα66 in MCF7 Breast Cancer Cells

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The expression of two human estrogen receptor-α (hERα) isoforms has been characterized within estrogen receptor-α-positive breast cancer cell lines such as MCF7: the full-length hERα66 and the N terminally deleted hERα46, which is devoid of activation function (AF)-1. Although hERα66 is known to mediate the mitogenic effects that estrogens have on MCF7 cells, the exact function of hERα46 in these cells remains undefined. Here we show that, during MCF7 cell growth, hERα46 is mainly expressed in the nucleus at relatively low levels, whereas hERα66 accumulates in the nucleus. When cells reach confluence, the situation reverses, with hERα46 accumulating within the nucleus. Although hERα46 expression remains rather stable during an estrogen-induced cell cycle, its overexpression in proliferating MCF7 cells provokes a cell-cycle arrest in G_0/G_1 phases. To gain further details on the influence of hERα46 on cell growth, we used PC12 estrogen receptor-α-negative cell line, in which stable transfection of hERα66 but not hERα46 allows estrogens to behave as mitogens. We next demonstrate that, in MCF7 cells, overexpression of hERα46 inhibits the hERα66-mediated estrogenic induction of all AF-1-sensitive reporters: c-*fos* and cyclin D1 as well as estrogen-responsive element-driven reporters. Our data indicate that this inhibition occurs likely through functional competitions between both isoforms. In summary, hERα46 antagonizes the proliferative action of hERα66 in MCF7 cells in part by inhibiting hERα66 AF-1 activity. (*Endocrinology* 146: 5474–5484, 2005)

GROWTH AND DIFFERENTIATION of the female reproductive tracts are under the critical influence of estrogens such as 17β-estradiol (E_2) (1, 2). It is well established that the mitogenic actions of these steroids also have critical influences on the etiology and progression of human breast and uterus cancers (3, 4). Normal and pathological growth-promoting effects of E_2 are achieved through stimulating cells in G_0 phase to enter the cell cycle and hastening the G_1 to S phase transition (5). Estrogens actions are exerted through specific receptors, the estrogens receptors (ER)-α (NR3A1) and -β (NR3A2) (6–8). Targeted disruption of ERα and ERβ genes clearly demonstrated that the postnatal development of uterus and mammary glands rely on ERα rather than ERβ (9). Furthermore, ERα expression is intimately associated with breast cancer (10, 11). E_2 stimulates the proliferation of breast cancer cells that express ERα, and ERα-positive tumors are more differentiated and have less metastatic potential than ERα-negative tumors. ERα is therefore used as a prognosis factor and is targeted in therapies aiming to cure E_2-dependent cancers. The specific functions of ERβ in breast cancers are not precisely known. However, this protein is detected in human breast cancer and, notably, exhibits a decreased expression in invasive breast tumors *vs.* normal tissues (12).

ERα belongs to the nuclear receptor superfamily of transcription factors, structurally organized in six functional domains (A to F) (13). The C domain is necessary and sufficient for the specific binding of the receptor to DNA. The E domain allows hormone binding, an event that induces specific conformational changes within the receptor. This three-dimensional remodeling allows ERα to modulate the transcriptional activity of target genes through two transactivation functions (AFs), AF-1 and AF-2, located in the B and E domains, respectively. The respective contribution that AF-1 and AF-2 make toward the activity of the full-length ERα is both promoter and cell specific (13–16). Accordingly, promoter and cell contexts can be defined as AF-1 or AF-2 permissive, depending on which AF is principally involved in ERα activity. Transcriptional modulation of E_2-target genes involves recruitment of ERα either directly through interaction with cognate DNA sequences [estrogen-responsive elements (EREs)], or protein/protein interaction with other transcriptional factors (17). ERα-mediated transactivation is then achieved through an ordered sequence of interactions established between the AFs and coactivators such as: 1) members of the p160 subfamily (exemplified by steroid receptor coactivator-1 and transcription intermediary factor-2); 2) cAMP response element binding protein-binding protein/

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Abbreviations: AF, Activation function; BrdU, 5-bromo-2′-deoxyuridine; ER, estrogen receptor; ERE, estrogen-responsive element; FACS, flow cytometry analysis; FCS, fetal calf serum; GRE, glucocorticoid receptor element; hERα, human estrogen receptor-α; IFA, immunofunctional assay; NP-40, Nonidet P-40; 4-OHT, 4-hydroxytamoxifen.

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p300; 3) complexes of the Srb-Med coactivator complex/thyroid hormone receptor-associated proteins/vitamin D receptor-interacting proteins/activator recruited cofactor class; and 4) AF-1-specific coactivators such as p68 and p72 RNA helicases (18–20).

Corroborating the role that estrogens have as mitogen, the expression of genes involved in the control of cell proliferation such as cyclin D1 (21), c-*fos*, c-*myc* (22, 23), or growth factor genes (IGF-I) (24) are under ERα control. Besides its transcriptional functions, ERα also presents nongenomic actions. For instance, ERα stimulates rapidly the Src kinase and MAPK pathways to trigger cell cycle progression (25).

An isoform of ERα, 46 kDa in size [human estrogen receptor-α (hERα)46], encoded by an mRNA variant was identified in MCF7 human breast cancer cells in which it is coexpressed with the full-length ERα (hERα66) (26). The importance of this isoform is illustrated by the observation that 50% of ERα mRNA encode hERα46 in osteoblasts (27). Expression of the hERα46 isoform was also reported in endothelial cells (28, 29). hERα46 lacks the N-terminal A and B domains and is consequently devoid of AF-1 (26). Mechanistically, hERα46 induces the transcription of an ERE-derived reporter gene construct only in AF-2-permissive cell contexts (26). In contrast, this naturally occurring truncated hERα is unable to transactivate the same reporter gene construct in cellular contexts in which AF-1 is the primary AF involved in hERα activity. Moreover, when both isoforms are coexpressed, hERα46 efficiently suppresses the AF-1 activity of hERα66 in a cell-specific context (26). Finally, unliganded hERα46 efficiently represses the transcription of target genes, this effect being reversed after E_2 binding (30, 31).

To date, no information exists on the exact function of hERα46 in epithelial breast cancer cells. Exhibiting functional properties different from those of hERα66, we hypothesized that the hERα46 may have a role to play in the control of ERα-positive breast cancer cell proliferation.

Materials and Methods

Plasmids

The reporter plasmids ERE-TK-Luc, hC3-Luc, and pCMV-β-Gal internal control have been previously described (32). The c-*fos*-Luc and cyclin D1-Luc reporter genes were obtained by inserting human genomic PCR products (−730/+41 and −205/+54, respectively) into pGL3-basic (Promega, Charbonnier, France). The reporter plasmid $(E/GRE)_2$-Luc was obtained by inserting two annealed oligonucleotides in the pGL3-promoter vector (Promega): [5′-CCGGGAAAGGGCAGACTGTTCTTGGATCCAAGGGCAGTCTGTTCTTTAAGCTTATA-3′] and [5′-GATCTATAAGCTTAAAGAACAGACTGCCCTTGGATCCAAGACAGTCTGCCCTT-3′]. Expression vectors pCR hERα66, pCR hERα46, and pCR $hER\alpha66_{GR}$ were generated by cloning the coding region of hERα66 (+228/+2030), hERα46 (+727/+2030), and $hER\alpha66_{GR}$ (HE82; generously provided by P. Chambon, IGBMC, Illkirch, France) into the pCR 3.1 vector (Invitrogen, Cergy-Pontoise, France). Inducible expression vectors pIND hERα66 and pIND hERα46 were prepared by cloning corresponding open reading frame into the pIND vector (Invitrogen). Ecdysone-mediated expression of these open reading frames was performed using the pVgRXR vector (Invitrogen).

Cell culture and transfections

Hela, HepG2, and MCF7 cells were maintained in DMEM (Invitrogen) supplemented with 5% fetal calf serum (FCS; Sigma, St. Quentin Fallavier, France), penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin (35 μg/ml) at 37 C in 5% CO_2. PC12 cells were cultivated in DMEM/F12 containing 7.5% charcoal dextran-treated FCS and 2.5% charcoal dextran-treated horse serum.

Stably transfected MCF7 clones, MCF7 pIND, MCF7 pIND hERα66, and MCF7 pIND hERα46, were obtained by transfecting MCF7 cells with pVgRXR plasmid and corresponding expression vectors with FuGENE 6 reagent (Roche, Meylan, France), and selection with 0.8 mg/ml G418 and 0.8 mg/ml zeocin (Invitrogen). Stably transfected PC12 cell lines, PC12 pCR3.1, PC12 hERα66, and PC12 hERα46, were obtained by transfecting PC12 cells with corresponding pCR3.1 expression vectors and selection with 0.8 mg/ml G418 (Invitrogen).

Transient transfections were performed with the FuGENE 6 transfection reagent (Roche) as previously described (33). After either 12 h (for ERE-controlled reporter gene analysis) or 48 h (for c-*fos* and cyclin D1-Luc reporter analysis), cells were washed and then treated for 36 h (ERE-controlled reporter) or 12 h (c-*fos* and cyclin D1-Luc reporters) with ethanol (vehicle control), 10 nM E_2, or 2 μM 4-hydroxytamoxifen (4-OHT). Luciferase and β-galactosidase activities were assayed on cell extracts.

Flow cytometry analysis (FACS) and [^{3}H]thymidine incorporation assay

Cells growing in 10-cm-diameter dishes were pulse labeled with 1 mM 5-bromo-2′-deoxyuridine (BrdU) for 3 h. After trypsinization, cells were collected in PBS containing 30% immunofunctional assay (IFA) buffer [10 mM HEPES (pH 7.4), 150 mM NaCl, 4% FCS, 0.1% NaN3], pelleted at 1000 rpm for 10 min, and fixed in 70% ethanol as previously described (34). Fixed cells were incubated in IFA buffer containing the α-BrdU-fluorescein isothiocyanate antibody (CALTAG Laboratories, Burlingame, CA) for 1 h at 4 C and then washed in IFA buffer including 0.5% Tween 20. These steps were omitted in control untreated samples. Finally, fixed cells were incubated in IFA buffer containing 100 μg/ml RNase A for 15 min at 37 C, and 25 μg/ml propidium iodide were added before analysis with a FACScan equipment (Becton Dickinson, Le Pont de Claix, France).

When assaying [^{3}H]thymidine incorporation, the cells were incubated with 0.6 μCi [^{3}H]thymidine 12 h before harvesting. Cells were then frozen and thawed, and incorporated [^{3}H]thymidine was collected on A filter papers using a 96-well harvester and quantified by β-counting.

Protein extracts

Subcellular fractionation was performed as described in the current protocol. Briefly, cells were harvested and resuspended in lysis buffer [10 mM Tris-HCl (pH 7.4), 3 mM CaCl2, 2 mM MgCl2] with protease inhibitors (Roche). Cells were then pelleted and incubated in Nonidet P-40 (NP-40) lysis buffer [10 mM Tris-HCl (pH 7.4), 3 mM CaCl2, 2 mM MgCl2, 0.5% NP-40, protease inhibitors] during 15 min. After centrifugation, the supernatant (cytoplasmic extract) was recovered, whereas the pellet (nuclei) was resuspended in radioimmunoprecipitation assay-lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS] containing protease inhibitors and sonicated (nuclear extract).

Western blotting

Twenty micrograms of proteins extracts were resolved on 10% SDS-PAGE and electrotransferred onto nitrocellulose membranes as previously described (26). Blots were incubated with the polyclonal anti-hERα HC20 (TEBU), the monoclonal anti-Lamin B Ab-1 (Oncogene, Boston, MA), or the monoclonal anti-β-actin AC-15 (Sigma) in PBS containing 0.1% Tween 20 and 5% nonfat milk powder for 1.5 h at room temperature. After washings, the blots were incubated with either a peroxidase-conjugated goat antirabbit (Pierce, Rockford, IL) or a peroxidase-conjugated goat antimouse (Pierce) for 1 h. Membrane-bound secondary antibodies were detected using the SuperSignal West Dura kit (Pierce) according to the manufacturer's instructions.

EMSA

In vitro transcription and translation were performed using the TNT-coupled reticulocyte lysate system as recommended by the manufacturer (Promega) with pCR 3.1, pCR hERα66, and pCR hERα46 used as

templates. Translation efficiency was checked by Western blot. Four microliters of rabbit reticulocyte lysate expressing ERα proteins were preincubated in gel shift assay buffer [10 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 100 mM KCl, 10% glycerol, 100 μg/ml BSA, 5 μg/ml of protease inhibitors, and 1 mM phenylmethylsulfonyl fluoride] with 2 μg of poly(dIdC) for 15 min at room temperature. The samples were then incubated for 15 min with decreasing concentrations (1–0.0625 ng) of radioactive oligonucleotide probe end labeled with [γ-^{32}P]ATP using T_4 polynucleotide kinase (Roche). Protein-DNA complexes were separated from free probes by nondenaturing electrophoresis on 5% polyacrylamide gels in 0.5× Tris-borate EDTA. The sequence of the 30-bp oligonucleotide used in these experiments is: 5′-ctgtgctcAGGTCAgacTGACCTtccatta-3′, with the consensus ERE sequence shown in *capital letters*.

Results

hERα46 is mainly located in the nucleus and its expression increases in confluent MCF7 cells

Aiming to further characterize functional differences between hERα66 and -46 isoforms, we first analyzed their respective subcellular localization during MCF7 cells growth, from scattered to confluent cells. During this time lapse, cell growth was monitored through cell numeration. Flow cytometry analysis was also used to evaluate the relative proportion of cells being in each of the different cell cycle phases (Fig. 1A). The percentage of MCF7 cells in S phase reaches its highest level 3 d after cell seeding and then progressively decreases until cells achieve confluence between d 9 and 12 (Fig. 1A). In parallel, Western blots performed on nuclear and cytoplasmic protein extracts probed the relative expression of either hERα isoforms in each compartment (Fig. 1, B and C). Antibodies against the Lamin B, a nuclear protein, controlled the efficiency of the fractionation, whereas β-actin was used as a loading control. Results indicate that hERα46 is almost totally localized in the nucleus and strongly accumulates in this compartment when cells reach confluency (Fig. 1B). In a few experiments, hERα46 was weakly detected in the cytoplasmic fraction at confluence. In contrast, hERα66 is localized in both the nucleus and cytoplasm, with a gradual accumulation observed during cell growth (until d 9, Fig. 1C).

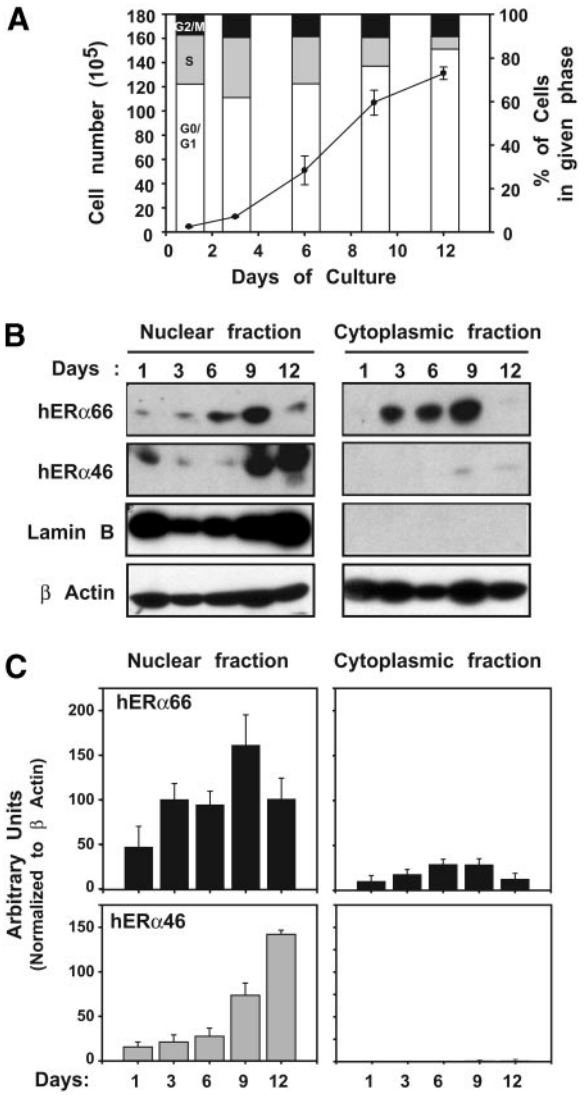

FIG. 1. hERα46 expression increases in hyperconfluent MCF7 cells. 10-cm-diameter dishes were seeded with 4×10^5 MCF7 cells in medium containing 5% FCS, and cells were harvested at different days of culture. A, Cell growth was monitored by cell numeration and DNA content determination through propidium iodide labeling and FACS analysis. Data represent the average ± SEM of three independent experiments. B, Western blot analysis of hERα66 and hERα46 levels in nuclear and cytoplasmic fractions of MCF7 cells harvested at the indicated days of culture. After subcellular fractionation, protein extracts (20 μg) were resolved on a SDS-polyacrylamide gel and subjected to immunoblotting using the anti-hERα HC20 antibody, anti-Lamin B Ab-1 antibody (fractionation control), and anti-β-actin AC-15 antibody (loading control). Representative data from three independent experiments are shown. C, The relative expression of hERα66 and hERα46 were quantified by densitometry analysis of the three experiments and normalized to β-actin signals. Values shown correspond to the average ± SEM of the three independent experiments.

hERα46 expression remains rather stable during estrogen-induced MCF7 cell cycle

The experiments depicted above might suggest the existence of a correlation between the expression pattern of hERα46 and specific phases of the cell cycle. To verify this hypothesis, we designed experiments aiming at analyzing the expression of hERα66 and hERα46 throughout an estrogen-induced cell cycle. To do so, 40% confluent MCF7 cells maintained in steroid-free medium [2.5% charcoal dextran-treated FCS] during 72 h were treated with 10 nM E_2 and synchronized in their cell cycle at the G_1/S phase transition using a 48-h aphidicolin treatment. Release of the aphidicolin block through washings then allowed the cells to progress throughout their cycle. The efficient completion of the synchronization step was confirmed by flow cytometry analysis, with 70% of the cells stopped in the G_1/S phase transition (Fig. 2A). Cells progressed through the S phase 6 h after aphidicolin withdrawal. At 9 h, cells went through the G_2/M phases and finally returned in an asynchronous state 12 h later (time point 24 h) with approximately 70% cells in G_0/G_1 phase (Fig. 2A). Assessing the relative distribution of either hERα isoforms within the nuclear and cytoplasmic fractions by Western blots showed that the nuclear amounts of hERα46 are stable up to S phase, slightly decrease during the G_2/M phases, and return to higher level when cells engage again in G_0/G_1 phases (Fig. 2B). In contrast, a strong decrease in nuclear and cytoplasmic hERα66 signals was observed

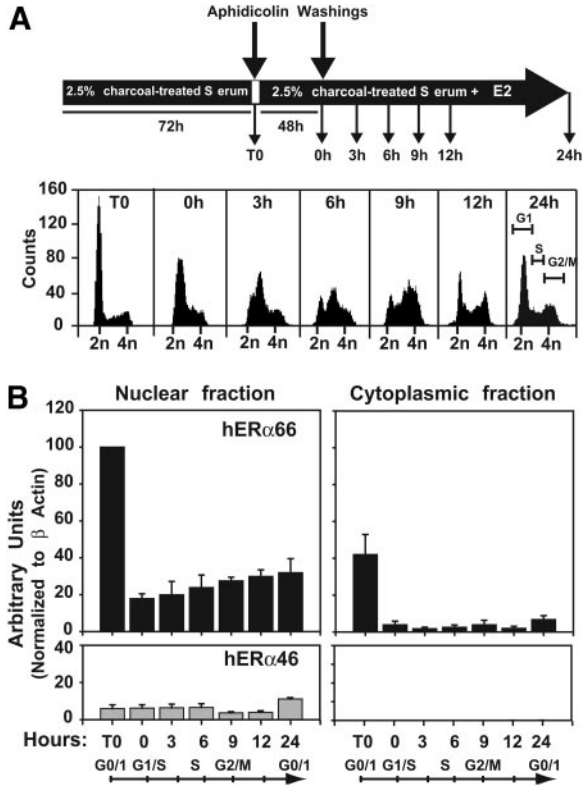

FIG. 2. hERα46 expression during estradiol-induced MCF7 cell cycle. Forty percent confluent MCF7 cells growing for 72 h in phenol red-free medium supplemented with 2.5% charcoal-treated FCS (T0) were synchronized at the G_1/S transition by a combined treatment with E_2 (10 nM) and aphidicolin (5 mg/ml) during 48 h. Cells were collected at the indicated time points after release from the aphidicolin blockade (time 0 h). A, After propidium iodide labeling, asynchronous (T0) and synchronized MCF7 cells were analyzed by flow cytometry. B, After subcellular fractionation, protein extracts (20 μg) were resolved on a SDS-polyacrylamide gel and subjected to immunoblotting with the anti-hERα HC20 antibody and the anti-β-actin AC-15 antibody (loading control). The relative expression of hERα66 and hERα46 were quantified by densitometry analysis of three independent experiments and normalized to β-actin signals. Values shown correspond to the average ± SEM.

during the G_1 phase after E_2 treatment. These expression levels remain repressed through the other phases of the cell cycle (Fig. 2B).

Together these data suggest that high levels of hERα46 are not found in quiescent MCF7 cells arrested in the G_0/G_1 phase but rather within MCF7 cells becoming refractory to growth, a state that is reached when cells are hyperconfluent.

Overexpression of hERα46 blocks MCF7 cells in G_0/G_1 phases

The above results likely suggest that hERα46 influences MCF7 growth. To confirm this assumption, MCF7 cell subclones (MCF7 pIND, pIND hERα66, and pIND hERα46) were established using ecdysone-inducible vectors expressing either hERα isoforms. After a 48-h treatment with 5×10^{-5} M ponasterone A, an ecdysone-like molecule, Western blots confirmed an inducible overexpression of the hERα46 isoform in growing MCF7 pIND hERα46 cells (Fig. 3A). In contrast, modifications of the hERα66 expression pattern

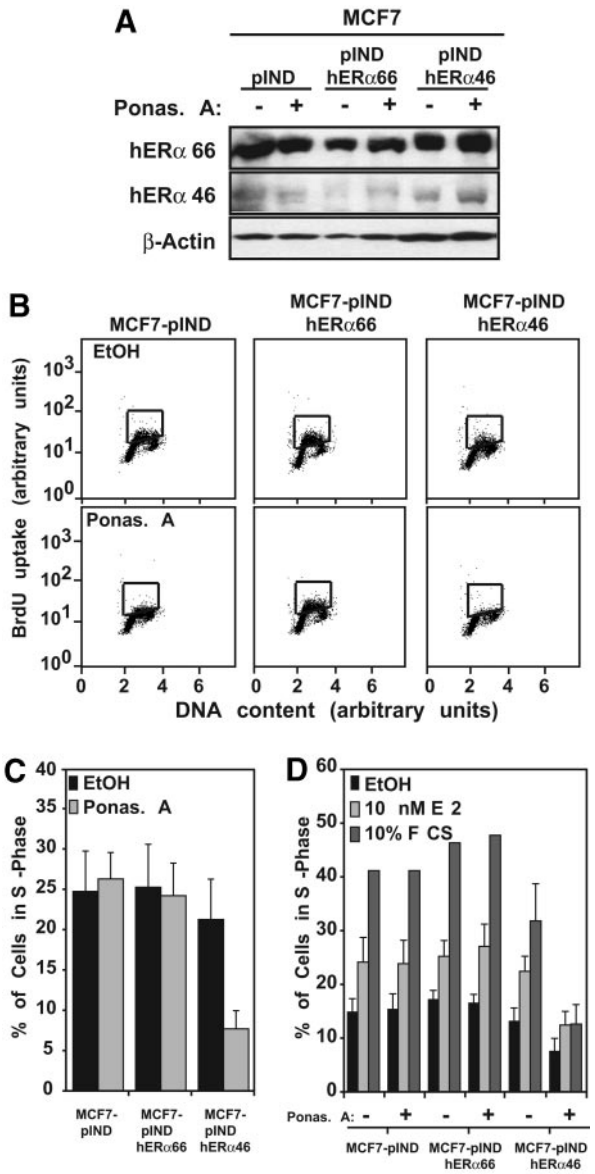

FIG. 3. Overexpression of hERα46 blocks MCF7 cell in G_0/G_1 phase. MCF7 cells were stably transfected with pVgRXR plasmid and pIND, pIND hERα66, or pIND hERα46 ecdysone-inducible expression vectors. Three clones, MCF7-pIND, MCF7-pIND hERα66, and MCF7-pIND hERα46 were selected as described in *Materials and Methods*. A, Western blot analysis probing the expression of both hERα66 and -46 forms in MCF7-pIND clones treated or not with an ecdysone-like molecule, ponasterone A (Ponas. A, 5×10^{-5} M), during 48 h. B, MCF7-pIND clones growing in normal medium complemented with 5% FCS were treated or not with 5×10^{-5} M Ponas. A during 48 h, and the cycle phase distribution of cell populations were determined by a dual BrdU/propidium iodide pulse labeling and flow cytometry analysis. C, Graphic representation of the percentage of cells in S phase deduced from B. Values are the average ± SD of four independent experiments. D, Consequences of a Ponas. A treatment on E_2- or FCS-induced cell proliferation of MCF7-pIND clones. MCF7-pIND, MCF7-pIND hERα66, and MCF7-pIND hERα46 were grown for 3 d in phenol red-free medium supplemented with 2.5% charcoal-treated FCS before being treated or not with either 10 nM E_2 or 10% serum during 24 h. Ponas. A treatment was or not performed during the last 48 h before harvesting. The percentage of cells in S phase was determined by propidium iodide labeling and FACS analysis.

were not apparent in the pIND hERα66 subclone after ponasterone A treatment. This is likely because of the particularly high levels of endogenous hERα66 already present in MCF7 cells. Similar results were also observed in MCF7 subclones stably transfected with vectors directing a constitutive expression of hERα66 (data not shown). Consequences of ponasterone A-driven expression of either hERα isoforms were first assessed on 40% confluent MCF7 cells growing in normal medium (5% FCS; Fig. 3, B and C). Flow cytometry analysis clearly demonstrated that ponasterone A specifically decreased the population of MCF7 pIND hERα46 cells in S phase by 65%, compared with untreated cells (Fig. 3C). Furthermore, treatment with ponasterone A specifically induced the accumulation of MCF7 pIND hERα46 cells in the G_0/G_1 phase of their cell cycle. These results were confirmed on another series of MCF7 pIND hERα66 and hERα46 subclones (data not shown).

The impact of a ponasterone A-induced expression of hERα46 on E_2-induced cell proliferation was subsequently analyzed. MCF7 subclones were maintained in medium complemented with 2.5% charcoal-treated FCS during 72 h prior treatment or not with 10 nM E_2 or 10% serum for 24 h. Subsequent flow cytometry analysis showed that the specific overexpression of hERα46 abolishes the hormonal stimulation of MCF7 growth, with this repressive effect occurring in the absence or presence of E_2 (Fig. 3D). Altogether, these experiments demonstrate that an overexpression of the hERα46 isoform affects MCF7 growth, mainly leading to a G_0/G_1 phase arrest.

In contrast to hERα66, hERα46 does not mediate estrogen-induced cell proliferation

The question of whether hERα46 may mediate cell proliferation induced by estrogen was next addressed. To reach this aim, we first had to select a cell line in which stable expression of hERα66 provokes E_2 to exhibit mitogenic effects. The establishment of such a system remained critical because estradiol treatment often inhibits rather than stimulates the growth of ERα-negative cell lines stably transfected with the ERα66 cDNA, in contrast to the situation observed in ERα-positive breast carcinomas (35). Among the different cell lines tested, PC12 cells gave the expected response, with E_2 having no impact on PC12 growth (PC12 control) and stimulating proliferation of PC12 cells stably expressing the hERα66 cDNA (PC12 hERα66). The PC12 cell line was therefore selected as biological system to probe the capability of hERα46 to mediate the mitogenic activity of estrogens. Stable transfection of hERα46 in PC12 cells did not confer an estradiol-induced cell proliferation, in contrast to the 2-fold increase in thymidine incorporation observed in PC12 hERα66 cells (Fig. 4). These results demonstrate that hERα46 is unable to mediate mitogenic activity of estrogen, in contrast to hERα66.

Overexpression of hERα46 inhibits the estrogenic induction of AF-1 permissive target genes in MCF7 cells

hERα46 is a potent ligand-inducible transcription factor in promoter and cell contexts sensitive to hERα AF-2 but has no transcriptional activity and behaves as a powerful inhibitor

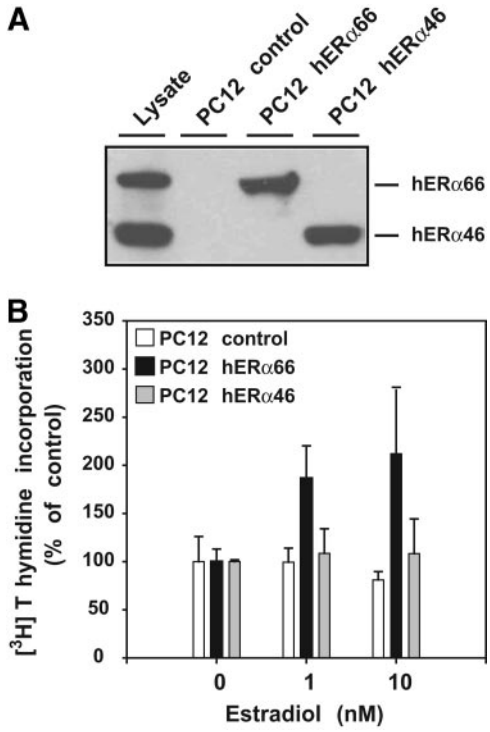

FIG. 4. hERα46 does not mediate estrogen-induced cell proliferation in PC12 cells. After transfection with pCR 3.1, pCR hERα66, or pCR hERα46 expression vectors, three stable PC12 clones, PC12 control, PC12 hERα66, and PC12 hERα46 were selected. A, Western blot analysis probing the expression of hERα66 and hERα46 forms in PC12 clones. B, Effects of E_2 on [^{3}H]thymidine incorporation into PC12 clones. Cells seeded in 24-well plates (5×10^4 cells/well) with DMEM/F12 containing 7.5% charcoal dextran-treated FCS and 2.5% charcoal dextran-treated horse serum were maintained for 2 d in the presence of the indicated concentration of E_2 before assessing [^{3}H]thymidine incorporation on the final day of culture. Values shown correspond to the average ± SD of three experiments.

of hERα66 activity in contexts in which AF-1 predominates over AF-2 (26, 33). The consequences of an increased expression of hERα46 on estrogen target gene activity will therefore depend on the relative permissiveness of MCF7 cells and target genes to hERα AF-1 and AF-2. The transcriptional properties of hERα46 were thus evaluated on reporter constructs placed under the control of different E_2-sensitive promoters. Taking into account the divergent roles that hERα isoforms have on E_2-mediated cell proliferation, we first selected promoters from genes involved in this process, exemplified by c-Fos and cyclin D1. These genes are transcriptionally induced by hERα66 in an ERE-independent mechanism requiring a functional AF-1domain (21, 22, 36–38). In hERα-positive MCF7 cells, the transcriptional activity of both promoters is 2.5-fold up-regulated by E_2; and, importantly, increasing amounts of hERα46 strongly inhibits this estrogenic induction (Fig. 5). In contrast, increasing amounts of pCR hERα66 enhances the estrogenic response of c-Fos promoter (Fig. 5A) and negatively impact cyclin D1 promoter activity only at the highest concentration (Fig. 5B). These results indicate that, in MCF7 cells, decreasing the hERα66 to -46 ratio by an overexpression of hERα46 inhibits the estrogenic induction of c-Fos and cyclin D1 promoters.

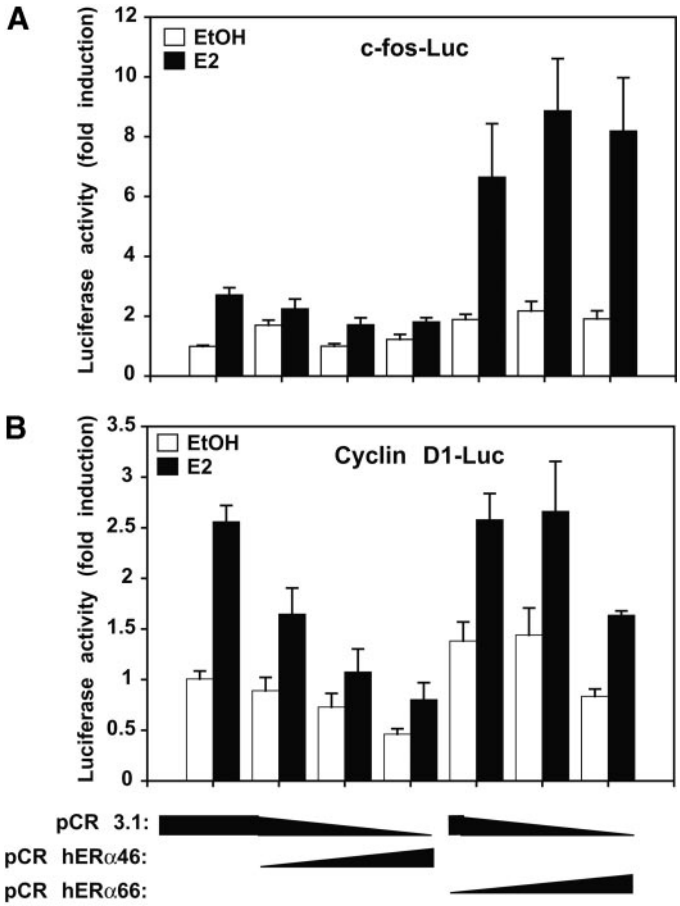

FIG. 5. Overexpression of hERα46 inhibits the estrogenic induction of the c-*fos* and cyclin D1 gene transcriptional activity in MCF7 cells. MCF7 cells, maintained in phenol red-free medium supplemented with 5% charcoal-treated calf serum, were transiently transfected with c-*fos*-Luc (A) or cyclin D1-Luc (B) reporter genes (200 ng) together with pCR 3.1 alone or with increasing quantity of pCR hERα46 or pCR hERα66 (50–200 ng). CMV-β-Gal (100 ng) was used as internal control. Forty-eight hours after transfection, cells were treated for 12 h with 10 nM E_2 or ethanol (EtOH). Luciferase activities were normalized with β-galactosidase activities and the values standardized to the reporter activity measured in the presence of pCR 3.1 alone without E_2. Values correspond to the average ± SEM of at least three separate transfection experiments.

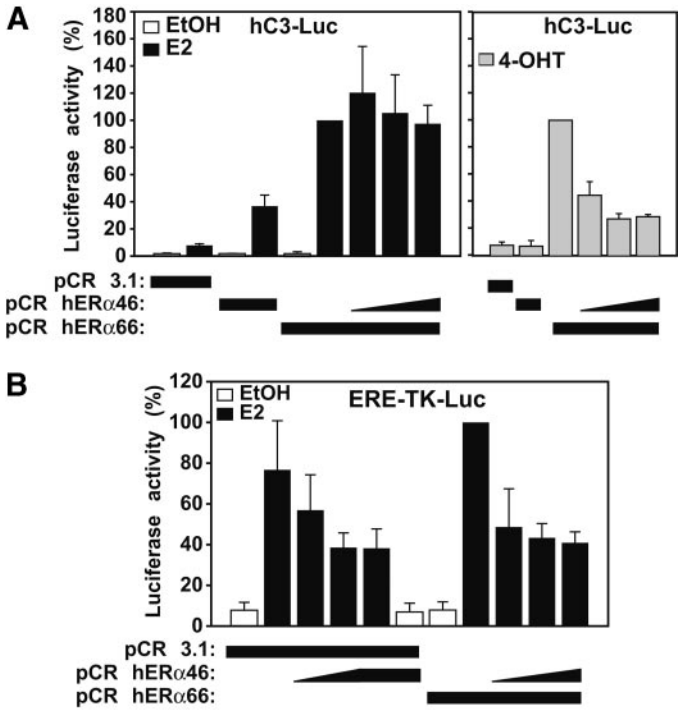

FIG. 6. hERα46 represses hERα66 AF-1 transcriptional activity on ERE-controlled genes in MCF7 cells. Two ERE-controlled reporter genes with no intrinsic preference for AF-1 and AF-2 hERα transactivation functions were selected for this study: the C3-Luc (A) and the ERE-TK-Luc (B). MCF7 cells, maintained in phenol red-free medium supplemented with 2.5% charcoal-treated calf serum, were transiently transfected with 200 ng of the reporter genes together with 50 ng of pCR 3.1 expression vectors (empty or encoding either hERα) alone or with increasing quantity of pCR hERα46 (0–200 ng). One hundred nanograms of CMV-β-Gal was used as internal control. Cells were treated for 36 h with 10 nM E_2, 2 μM 4-OHT, or ethanol (EtOH), as indicated within the panels. Luciferase activities were normalized with β-galactosidase activities, and results were expressed as a percentage of the reporter activity measured in the presence of the expression vector pCR hERα66 alone and E_2 (or 4-OHT). Values correspond to the average ± SEM of at least three separate transfection experiments.

To assay the generality of this observation, we subsequently analyzed the impact of increasing concentrations of hERα46 on the complement 3 promoter (C3-Luc), which contains an ERE and has no intrinsic preference for AF-1 or AF-2 (33). In the presence of E_2, hERα46 exhibited a 70% lower transactivation capability than hERα66 on this mixed AF-1/AF-2 reporter gene (Fig. 6A). Therefore, MCF7 cells are less sensitive to AF-2 than AF-1. Despite this prevalence of the MCF7 cell context toward AF-1, increasing amounts of pCR hERα46 had no effect on C3-Luc activation by hERα66 in the presence of E_2 (Fig. 6A). This contrasted with the expected inhibition of endogenous hERα66 activity occurring in strict AF-1-sensitive cell context. We therefore treated transfected MCF7 cells with 4-OHT, a partial hERα agonist whose estrogenic activity exclusively depends on AF-1, *i.e.* detectable only in cell and promoter contexts sensitive to AF-1 (14). Furthermore, the C3-Luc gene is a well-characterized 4-OHT-responsive reporter system (16). The 4-OHT-induced transcriptional activity of the C3-Luc gene was inhibited with increasing hERα46 expression (Fig. 6A). In these conditions, hERα46 thus behaves as an inhibitor of hERα AF-1 activity, revealing a cell-context mainly sensitive to AF-1. Analysis of the ERE-TK-Luc, the second reporter gene with no intrinsic preference for AF-1 and AF-2, seemed to confirm this assumption. In contrast to the C3-Luc reporter, the direct evaluation of the respective activities of either hERα isoforms was biased by the high activity of the ERE-TK-Luc reporter induced by endogenous hERα proteins (Fig. 6B). However, increasing amounts of exogenous hERα46 inhibited E_2-induced hERα66 transcriptional activity on this reporter gene, confirming the AF-1 permissiveness of MCF-7 cells.

Altogether, these results demonstrate that MCF7 cells are mainly sensitive to the AF-1 function of hERα, however, with a low permissiveness to AF-2. In such context, changes in the hERα66 to hERα46 ratio should mainly impact the transcriptional activity of AF-1-permissive estrogen target genes.

The hERα46 homodimer has more affinity for an ERE than a hERα66 homodimer

The ability of hERα46 to behave as an effective AF-1-negative competitor on ERE-controlled genes might result from its aptitude to compete for the binding of hERα66 to an ERE. We therefore assessed the ability of hERα46 to compete for the binding of hERα66 to an ERE in EMSAs. To do so, we produced *in vitro* rabbit reticulocyte lysate extracts containing constant levels of hERα66 proteins in conjunction with increasing amounts of hERα46, as verified in Western blots (Fig. 7A). Subsequent EMSAs revealed an ERE/hERα66 homodimer complex, a fast migrating ERE/hERα46 homodimer complex, and an intermediate ERE/hERα66/46 heterodimer complex. Interestingly, when little amounts of hERα46 are coproduced with the hERα66, it is the heterodimer complex that is preferentially formed; with the inverse also verified (Fig. 7A and data not shown). Importantly, increasing the amounts of hERα46 protein destabilized the ERE/hERα66 homodimer complex. These results might reflect differences in the respective affinity of the hERα isoforms dimers for an ERE. We thus followed the binding of each isoform to DNA with increasing quantities of radiolabeled ERE in EMSAs, and the results were next evaluated by Scatchard analysis (Fig. 7B). These experiments demonstrate that the hERα46 homodimer has a twice more potent intrinsic affinity for the ERE than does the hERα66 homodimer, with a calculated affinity constant of 0.11 and 0.2 nM, respectively. Unfortunately, the affinity of the hERα66/46 heterodimer for the ERE could not be defined by this approach due to the impossibility to produce protein extracts containing only the heterodimer.

In conclusion, with a 2-fold higher affinity for the ERE, the hERα46 dimer is able to compete the binding of the hERα66 homodimer and, by such means, would be able to inhibit the transcriptional activity of AF-1-permissive genes induced by the hERα66.

The hERα66/hERα46 heterodimer is AF-1 permissive

The ability of the hERα46 to act as an effective AF-1-negative competitor might also result from its ability to form

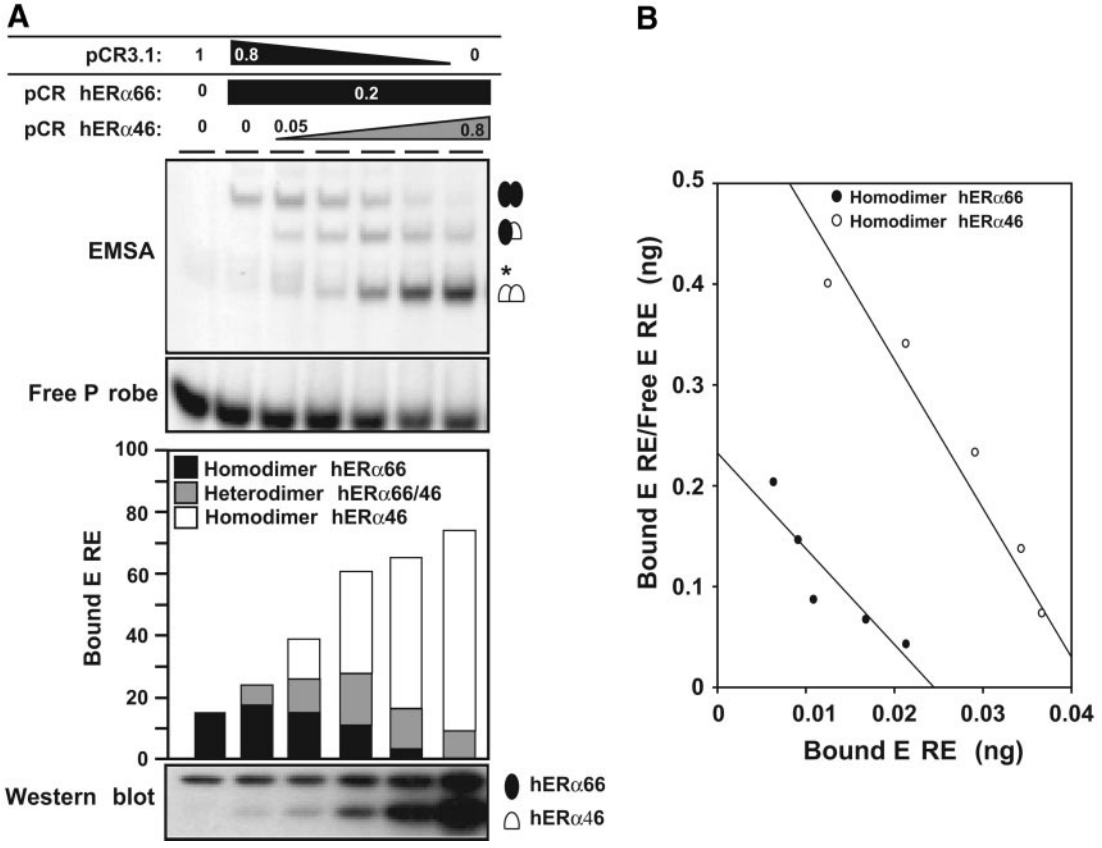

FIG. 7. hERα46 homodimer is more affine for an ERE than hERα66 homodimer. A, *In vitro* transcription/translation in rabbit reticulocyte lysate used plasmid mixes (completed to 1 μg with empty vector pCR 3.1) containing 0.2 μg pCR hERα66 and increasing amounts of pCR hERα46 (0, 0.005, 0.01, 0.02, 0.04, and 0.8 μg). Then 2.5 μl of *in vitro*-translated products were subjected to EMSA through incubation with 0.05 ng of ^{32}P-labeled ERE and resolved on a 5% polyacrylamide gel. The positions of the three specific complexes (ERE/hERα66 homodimer, ERE/hERα66/46 heterodimer, and ERE/hERα46 homodimer) are indicated. The relative amounts of these three complexes were then quantified by densitometry. Values correspond to the average of two independent experiments. In parallel, 2.5 μl of *in vitro*-translated products were resolved on a 10% SDS-polyacrylamide gel and then subjected to immunoblotting with the anti-ERα HC20 antibody. B, Plasmid samples (completed to 1 μg with empty vector pCR 3.1) containing either 0.2 μg of pCR hERα66 or pCR hERα46 were *in vitro* transcribed and translated in rabbit reticulocyte lysate. Then 2.5 μl of *in vitro*-translated products were incubated with an increasing amount of ^{32}P-labeled ERE (0.0625, 0.125, 0.25, 0.5, and 1 ng) and resolved on a 5% polyacrylamide gel. The amounts of homodimers were quantified and subjected to Scatchard analysis.

heterodimers with the hERα66. Because these heterodimers contain only one AF-1 region, we next assessed whether they might be inactive in cellular contexts strictly permissive to this transactivation function. However, the binding of both hERα homodimers and hERα66/46 heterodimer to EREs prevent the specific determination of the transcriptional activity of the hERα66/46 on ERE-containing reporters. To circumvent this, we set up a strategy similar to the one previously used by Tremblay *et al.* (39) when defining the transcriptional properties of the ERα/ERβ heterodimer. This method takes advantage of the mutation of three residues within the ERα DNA binding domain that change its DNA binding specificity to that of a glucocorticoid receptor (Fig. 8A) (40). This $hER\alpha_{GR}$ mutant induces transcription of a GRE-TK-Luc but not of an ERE-TK-Luc reporter gene (Fig. 8B). To measure the specific activity of the $hER\alpha_{GR}$/hERα46 and $hER\alpha_{GR}$/hERα66 heterodimers, we used a reporter gene whose transcription is under the control of two hybrid E/GRE DNA-responsive elements [$(E/GRE)_2$-SV-Luc]. Importantly, in strict AF-1 (HepG2) or strict AF-2 (HeLa) permissive cell lines, an E_2-induced transcriptional activity of this reporter gene occurred only when $hER\alpha_{GR}$ was coexpressed with either hERα66 or hERα46 (Fig. 8C). Similar results were obtained in MCF7 cells, with an induction of the reporter gene in the presence of E_2 observed when expressing only $hER\alpha_{GR}$ due to its heterodimerization with endogenous hERα. These results indicate that the hERα66/46 heterodimer is as potent as a hERα66 homodimer for activating transcription in both AF-2- and AF-1-permissive cell contexts. They also suggest that a single AF-1 region is sufficient for a hERα66 ho-

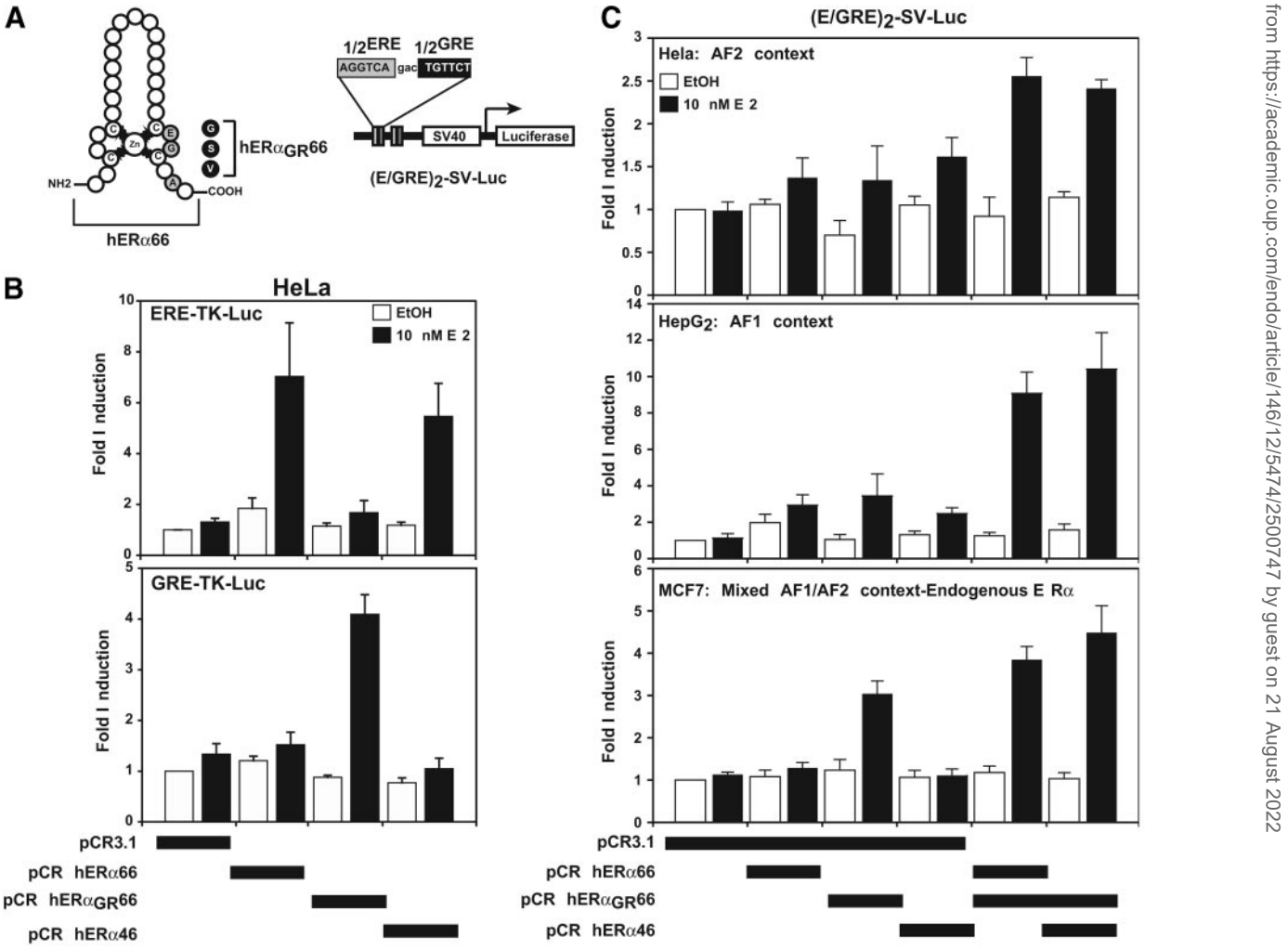

FIG. 8. An AF-1 activity is retained within the hERα66/hERα46 heterodimer. A, Schematic representation of the first zinc finger of the DNA binding domain of hERα. The positions of the three amino acids that contribute to DNA binding specificity are indicated. These residues were mutated to substitute the specificity of hERα66 binding to ERE for a specific binding to a GRE ($hER\alpha_{GR}66$ mutant). The structure of the $(E/GRE)_2$-SV-Luc reporter gene with its two hybrid E/GRE DNA-responsive elements is also indicated. B, HeLa cells were transfected with the ERE-TK-Luc or GRE-TK-Luc reporter genes (100 ng) in conjunction with pCR 3.1, pCR hERα66, pCR $hER\alpha66_{GR}$, or pCR hERα46 (50 ng). One hundred nanograms of CMV-β-Gal was used as internal control. Luciferase activities were normalized with β-galactosidase activities, and the results were standardized to the reporter activity measured in the presence of pCR 3.1 without E_2. Values correspond to the average ± SD of at least three separate transfection experiments. C, AF-2-permissive HeLa cells, AF-1-permissive HepG2, and MCF7 cells were transfected with 200 ng of $(E/GRE)_2$-SV-Luc reporter and pCR 3.1, pCR hERα66, pCR $hER\alpha66_{GR}$, or pCR hERα46 (50 ng) alone or in combination as indicated on the bottom of the graph. Results are expressed and normalized as in B.

modimer to function. The AF-1 dominant-negative action of the hERα46 on ERE-driven gene is therefore not a consequence of its ability to form a heterodimer with hERα66.

Discussion

The role of estrogens in the promotion and development of breast cancers was initially established by clinical and epidemiological observations, such as the therapeutical efficiency of ovariectomy and antiestrogen therapy. Moreover, E_2 has a potent mitogenic effect on ERα-positive breast cancer cell lines such as MCF7 cells (4, 10, 11). However, to date, the molecular mechanisms through which E_2 controls the growth of ERα-positive breast cancer cells are poorly understood. A first step toward the understanding of these processes was reached through the identification of an isoform of the hERα, hERα46, which is coexpressed with the full-length hERα66 in MCF7 cells (26). Being devoid of the A/B domain containing the AF-1, the hERα46 harbors specific functional properties (26). We hypothesized that hERα46 may influence the E_2-induced growth of MCF7 cells and therefore sought to determine whether a direct correlation exists between the expression of hERα46 and cell growth and to define the underlying mechanisms.

First, we show that during MCF7 cell growth, hERα46 is mainly expressed in the nucleus at levels remaining relatively low, whereas hERα66 accumulates in the nucleus and, to a lesser extent, in the cytoplasm, as previously reported (41). When cells reach hyperconfluency and become quiescent, the situation reverses, with a strong accumulation of hERα46 within the nucleus concomitant with a decrease in hERα66 levels. We have previously shown that the amounts of hERα46 present in whole-cell extracts are constant, when comparing confluent and nonconfluent (20% confluence) MCF7 cells (26). This apparent discrepancy with the present data are explained by the fact that the previous analysis used cells that just reached confluence, when hERα46 expression is still relatively low. As shown in Fig. 1, an accumulation of hERα46 within the nucleus requires the cells to be hyperconfluent. Consequently, when cells have reached confluency, the expression of hERα46 is obviously subject to additional controls, whose mechanisms remain to be defined.

Interestingly, this accumulation of hERα46 correlates with a stage when cells become refractory to E_2-induced growth. Indeed, several years ago, electrophoretic analysis of *in vivo*-labeled ER with ^{3}H-tamoxifen aziridine showed that the size of ER protein was dependent on cell confluency: whereas growing MCF7 cells expressed a monomeric binding entity of 62 kDa, hyperconfluent cells presented a 47-kDa binding entity (42). Furthermore, during the different phases of the estrous cycle, both entities coexist in distinct proportions during the diestrous (1/2) and proestrous (1/1). Importantly, only the smaller form was detected during the estrous phase, a phase that is associated with the uterus being refractory to E_2 stimulation (43). Altogether, these data suggest that high expression levels of ERα46 correlate with cells being refractory to the mitogenic effects of E_2.

Our experiments using an ecdysone-inducible system clearly show that an increase in hERα46 expression in nonconfluent MCF7 cells reduces the percentage of cells in S phase after estrogen or serum induction of cell growth. Other studies have shown that the permissiveness of osteoblast-like SaOS cells to E_2 mitogenic effects, obtained through the exogenous expression of hERα66, is altered in a dose-dependent manner by hERα46 (27). Therefore, hERα46 obviously behaves as a cell growth inhibitor when it is overexpressed in MCF7 cells, probably through controlling the proliferative influence of hERα66. To validate these conclusions, we used ERα-negative PC12 cell line, in which the stable expression of hERα66 but not hERα46 allows estrogen to mediate cell proliferation. This further indicates that the hERα A/B domains and probably its AF-1 activity are required for the receptor to exhibit a proliferative influence. Corroborating this result, Fujita *et al.* (44) previously reported that a fully activated AF-1 induces growth of ERα-positive breast cancers. In ERα$^{-/-}$ mice generated by an insertional disruption of the ERα gene in the first coding exon, critical E_2-induced growth deficiencies were observed in breast and uterus tissues (9). Although totally abolishing the production of the full-length ERα, this disruption does not suppress ERα46 expression (45). This further emphasizes the importance of AF-1 in ERα proliferative activity.

Mediation of estrogen-induced cell proliferation by hERα66 results in part from modifications in the expression patterns of genes, *e.g.* those involved in the control of the cell cycle such as c-*fos* and cyclin D1. Previous studies clearly demonstrated the importance of AF-1 activity in the estrogenic induction of these genes. Notably, a truncated hERα devoid of the A/B domain (HE19, equivalent to hERα46) did not transactivate the c-*fos* and cyclin D1 promoters (21, 36, 37). Extending these data, the present study clearly demonstrates that increasing expression of hERα46 in MCF7 cells abolishes the estrogenic induction of both of these promoters in a dose-dependent manner. In parallel, we determined MCF7 cells as providing an environment permissive to both AFs, with nevertheless an increased sensitivity to AF-1. In these cells, AF-2-permissive reporter genes such as pS2-Luc (data not shown) are equally sensitive to both hERα isoforms, and increasing the amounts of hERα46 does not impact hERα66 transcriptional activity. In contrast, hERα46 inhibited the transcriptional activity of hERα66 on AF-1-sensitive genes in a dose-dependent manner. Consequently, changes within the respective levels of expression of hERα isoforms as occurs when cells reach confluence should specifically inhibit hERα66-mediated transcription of E_2 target genes sensitive to AF-1 but not AF-2. These data are particularly relevant because the proliferative activity of hERα66 seems to be mediated, as previously mentioned, by its AF-1 activity.

Interestingly, hERα46 shares several functional similarities with ERβ. For instance, both of these ER forms are devoid of the AF-1 present in hERα66, although sharing relatively conserved DNA and ligand binding domains (7, 26). Consequently, hERα46 and ERβ induce the transcription of ERE-driven genes mainly via their AF-2 (26, 46). Recent studies also showed that, as does hERα46, ERβ counteracts the activity of ERα66 in many cellular systems. Indeed, the stable expression of ERβ inhibits the E_2-stimulated proliferation of the ERα-positive MCF7 or T47D breast cancer cells (47, 48). Furthermore, unlike ERα66, ERβ represses cyclin D1 gene transcription and blocks ERα66-mediated induction when

both receptors are present (38). Finally, the expression of ERβ decreases in invasive breast cancers tissues, compared with adjacent normal mammary gland (12), suggesting that the ERα66 to ERβ ratio increases during carcinogenesis. Correspondingly, the highest ERα66 to ERα46 ratios are observed in growing MCF7 breast cancer cells and the lowest in hyperconflent MCF7 cells being refractory to E_2 mitogenic effect or in primary human cultures from vascular endothelial cells (28, 29) or osteoblasts (27). Although the specific functions of ERα46 and ERβ in cancer are not known, there is increasing evidence that these ER proteins deficient in AF-1 have inhibitory effects on cellular proliferation.

Several mechanisms might explain the ability of hERα46 to efficiently suppress the AF-1 activity of hERα66. First, hERα46 may compete the binding of hERα66 to ERE or other transcription factors (AP-1 and Sp1 proteins) in ERE-independent mechanisms. Indeed, both forms efficiently bind EREs and physically interact with AP-1 and specificity protein 1 (49, 50). We show in this report that, *in vitro*, increasing amounts of hERα46 squelches the binding of hERα66 to ERE. As determined by Scatchard analysis, this competition is facilitated by a 2-fold increased affinity of the hERα46 for an ERE, compared with the hERα66 homodimer. This is in accordance with previous studies ascribing a better affinity of receptors deleted from their N-terminal A/B domains for their hormone-responsive elements (51, 52). For instance, deletion of the A/B domain from the *Xenopus* ERα increases by 2-fold its affinity for an ERE (52).

EMSAs using *in vitro*-translated proteins also revealed that hERα46 heterodimerizes with hERα66, generating a protein complex that has only one AF-1 function. Because this would provide a mean for hERα46 to inhibit the AF-1 of its partner, we evaluated whether the AF-1 domain of hERα66 is still functional when heterodimerized with hERα46. To specifically monitor the transactivation properties of the heterodimer, we used a hERα66 mutant ($hER\alpha66_{GR}$) that specifically binds glucocorticoid receptor elements (GREs) (40). Expression of this mutant together with hERα46 results in the formation of a $hER\alpha66_{GR}$/hERα46 heterodimer whose specific activity was assayed on a reporter gene placed under the control of a hybrid E/GRE-responsive element. The heterodimer efficiently activated the reporter gene in AF-2-sensitive cells such as HeLa cells but, surprisingly, also in strictly AF-1-permissive HepG2 cells. This means that heterodimerization with hERα46 does not impact on the activity of hERα66 mediated by its AF-1. Interestingly, within the ERα/ERβ heterodimer, each AF-1 domain can be activated independently (39). This demonstrates that ERα AF-1 retains its transcriptional properties within the context of ERα/ERβ and hERα66/hERα46 heterodimers and suggests that only one AF1 domain is sufficient for ERα to function.

We conclude from these results that the AF-1 dominant-negative action of hERα46 is not due to an inhibition of the AF-1 activity within a hERα66/46 heterodimer. Whereas a transcriptional activity of the hERα66/46 heterodimer was detected in MCF7 cells using the $hER\alpha66_{GR}$ mutant, we failed to detect the presence of endogenous heterodimers in these cells by coimmunoprecipitation experiments (data not shown), suggesting that hERα46 more readily homodimerizes than heterodimerizes with hERα66 in MCF7 cells.

The accumulation of hERα46 in the nucleus during MCF-7 cells growth arrest can inhibit the activity of hERα66, at least through competition for the binding to a shared ERE. Besides this passive mechanism, an active process can also be envisioned, in which the substitution of hERα66 by hERα46 on the ERE would direct the specific recruitment of corepressors. Indeed, in contrast to the hERα66 that interacts with recruitment of corepressors only when liganded to antiestrogens such as 4-OHT, the hERα46 isoform can recruit these cofactors in the absence of any ligand (30, 31). However, this hypothesis would imply that a fraction of the large amounts of hERα46 produced when cells reached confluence stays unliganded. This remains to be determined.

When MCF-7 cells reach confluence, some of the intracellular hERα46 is detected in the cytosolic fraction. This suggests that the mediation of cell growth arrest by hERα46 can also involve the activation or the inhibition of nongenomic pathways. In vascular endothelial cells, a pool of hERα46 was found associated with cell membrane in a palmitoylation-dependent manner (28, 29). In these cells, hERα46 modulates the actions of estrogens initiated at the level of the cell membrane. As an example, hERα46 activates the endothelial nitric oxide synthase pathway more efficiently than hERα66 (28, 29). Although we did not succeed in identifying a pool of hERα46 associated with MCF7 cells membrane (data not shown), the occurrence of specific nongenomic regulations initiated by hERα46 in MCF-7 cells cannot be ruled out.

In conclusion, the generation of hERα46 proteins in mammary cells constitutes a key regulatory element in the estrogenic control of cell growth. Actions of hERα46 are obviously mediated in part through genomic effects by interfering with the transcriptional activity of hERα66. Further studies are now required to identify genes whose transcription is placed under the specific control of either hERα isoforms.

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