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Trophic effect in MCF-7 cells of ERα17p, a peptide corresponding to a platform regulatory motif of the estrogen receptor α—Underlying mechanisms

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Abstract

As yet, estrogen receptor α (ERα) inhibitors used in clinical practice target a unique site, i.e. the hormone-binding pocket. With the aim of discovering other potential therapeutic targets in the receptor, we studied its AF-2a domain, a site that proves to be critical for ligand-independent ERα activity. Previous studies from our laboratory highlighted an auto-inhibitory action associated with a site included in this domain, i.e. the P_{295}-T_{311} sequence. Accordingly, a deletion of this sequence produces a constitutively activated receptor mutant. More interestingly, a synthetic peptide with the P_{295}-T_{311} sequence (ERα17p) elicits in breast cancer cell lines estrogenic responses that may be ascribed to a competitive mechanism towards the P_{295}-T_{311}-associated auto-inhibition of ERα. In the present study, we show that ERα17p sustains MCF-7 cell growth in estrogen-depleted culture medium by inducing molecular events promoting G1/S phase transition. We demonstrate, moreover, that this proliferative activity is associated with receptor down regulation (acceleration of ERα degradation and repression of ESR1 gene transcription), similar to that induced by estrogen agonists. Complementary studies suggest that our observations may be, at least in part, relevant to a competitive inhibition affecting ERα–Hsp70 association. Hence, the design of drugs able to stabilize ERα–Hsp70 complexes – where the receptor is in an inactive conformation – may be of therapeutic value.
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Keywords: Breast cancer; Estrogen receptor α; Hsp70; ERα17p; Synthetic peptide

1. Introduction

Estrogen receptor α (ERα) is the key mediator of breast cancer cell response to estrogens and antiestrogens. It is a member of the superfamily of nuclear receptors, a class of ligand-activated transcription factors sharing a common structure characterized by five functional domains (i.e. A/B, C, D, E and F domains) [1]. Estradiol (E_2) binding induces a change in ERα conformation, promoting the interaction of the receptor with specific DNA sequences located within the promoter region of target genes (estrogen response elements; EREs) and the recruitment of coregulatory proteins. Ligand-induced recruitment of coregulators mostly occurs at a small site named activation function-2 (AF-2) located in the C-terminal edge of the hormone-binding

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domain (HBD; E domain) [2]. By contrast, the A/B domain of ERα contains the activation function-1, i.e. AF-1, which acts independently of ligand-binding [3]. In addition, an autonomous activation function, i.e. AF-2a, has been localized in the N-terminal region of the LBD [4]. Besides, ERα itself may also operate as a coregulator for other transcription factors such as AP-1 and Sp1 [5–7].

Involvement of ERα in the proliferation of breast cancer cells has been known since the early 1970s [8] and has led to the design of a variety of drugs aimed at counteracting estrogen-mediated signaling (i.e. antiestrogens). However, all antiestrogens which have been developed so far function as competitive antagonists, acting via their association with the estrogen binding pocket of the receptor. Because some tumors which express ERα are, or can become resistant to these drugs [9], molecules aimed at targeting other domains of the receptor and/or of coregulators might prove to be of therapeutic value. In support to this view, recent investigations have disclosed interesting properties in peptides containing a canonical *LxxLL* sequence similar to the ERα-interacting domain in coactivators (see Ref. [10] for review). Indeed, a few of such peptides, as well as some non-peptidic mimics – called PERMs for *"peptidomimetic estrogen receptor modulators"* [11] or CBIs for *"coactivator binding inhibitors"* [12] – have been shown to modulate ERα activity by interacting with the receptor AF-2 domain [11–19].

Our own investigations focus on the ERα AF-2a domain because it represents a regulatory platform of major importance for the regulation of ERα activity. This functional domain indeed contains key aminoacids subjected to phosphorylation [20,21], acetylation [22,23], sumoylation [24] and monoubiquitination [25,26]. Moreover, it is directly implicated in the association of the ERα with calmodulin [27–30], association which contributes to enhance receptor-mediated transcription (see Ref. [31] for review) and inhibits E6AP-mediated ubiquitination [32]. Of note, a nuclear localization signal, which is also a proteolytic site [33–35], has also been reported in this part of the receptor.

With the hope of interfering with ERα mode of action, we synthesized a peptide corresponding to the putative calmodulin-binding motif in the receptor (ERα17p; P_{295}LMIKRSKKNSLALSLT$_{311}$) [30,36]. Amazingly, we found that exposure of breast cancer cells to this peptide provokes estrogenic responses. Thus, we showed that ERα17p stimulates ERα-dependent transcription and enhances the proliferation of various ERα-positive breast cancer cell lines, while it is inactive on their ER-negative counterparts. The finding that the deletion of the P_{295}-T_{311} sequence resulted in a constitutively active receptor led us to conclude that this motif possesses a repressive function through intra- and/or intermolecular interaction(s). In this context, it should be stressed that the presence of an inhibitory motif has been recently localized in the hinge region (D domain) of the androgen receptor [37].

A computer-assisted approach has generated a model accounting for our observations. Within the HBD, we identified a putative regulatory interaction between a segment of the P_{295}-T_{311} motif and the β-turn/H4 region [38] located in the neighborhood of H3-H5, a region known to recruit coregulators [39,40]. Hence, the estrogen-like activity of ERα17p might result from its ability to abrogate an auto-inhibitory folding stabilized, in part, by the P_{295}-T_{311} motif. The potential dissociation of protein(s) contributing to the maintenance of this inactive form (coinhibitors and/or chaperones) in favor of the association of a coactivator may also be advocated. In agreement with this hypothesis, we demonstrate here that ERα17p inhibits the binding of Hsp70 to the receptor.

Trophic action of estrogens on breast cancer cells is commonly associated with a progressive ERα loss [41,42]. In the present study we show that the stimulatory effect of ERα17p on MCF-7 cell growth is also associated with such a loss. According to our data, dissociation of the ERα–Hsp70 complex induced by the peptide may be a key step in this phenomenon.

2. Material and methods

2.1. Chemicals

ERα17p and ERα17p-biotin were produced as previously described [30,36] in the Natural Substances Laboratory of Meurice Institute according to the Atherton and Sheppard solid phase peptide synthesis method [43] on an Advanced ChemTech 90 apparatus. Estradiol (E_2), 4-hydroxytamoxifen (OH-Tam), cycloheximide (CHX), phenylmethylsulfonyl fluoride (PMSF), IGEPAL (NP-40) and agarose-bound anti-rabbit and anti-mouse IgG antibodies were from Sigma (St Louis, MO). MG-132 was obtained from Calbiochem (San Diego, CA). Tosyl-L-phenylalaninechloromethylketone (TPCK) came from Roche Diagnostics (Mannheim, Germany). Fulvestrant (ICI 182,780) was purchased from Tocris (Ellisville, MO). L-[^{35}S]methionine (>1000 Ci/mmol) was from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Streptavidin–agarose beads UltraLink Immobilized Streptavidin Plus and peroxidase-labeled secondary antibodies were supplied by Pierce (Rockford, IL). Anti-ERα antibodies F-10 and HC-20 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin MAB1501R antibody was from Chemicon (Temecula, CA). Anti-Rb, phospho-Rb 780, 795 and 807/811, anti-p21 (Cip1/Waf1), anti-p27 (Kip1), anti-p15 (INK4B) and anti-Hsp70 antibodies all came from Cell Signaling Technology (Danvers, MA).

2.2. Cell culture

MCF-7 breast cancer cells were propagated at 37 °C (5% CO_2, humid atmosphere) in Earle's based minimal essential medium (EMEM) supplemented with Phenol Red, 4 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% heat-inactivated fetal bovine serum (FBS) (all reagents from Invitrogen; Carlsbad, CA). Experiments were performed in EMEM without Phenol Red and containing 10% charcoal-stripped FBS. The change of medium was performed 48 h before treatment, except for growth measurement and cell cycle analysis by flow cytometry (24 h).

2.3. Cell growth measurement

MCF-7 cells seeded in 96-well plates (3000 cells/well) were treated with ERα17p at 10 μM and/or E_2 at 0.1 nM, OH-Tam at 1 μM or fulvestrant at 1 μM for 24, 48 and 72 h. Cell growth was measured by crystal violet staining [44] as previously described [30]. Briefly, cell cultures were washed with PBS, fixed with 1% (v/v) glutaraldehyde in PBS and stained with 0.1% (w/v) crystal violet. After removal of excess dye, cell-bound crystal violet was extracted with 1% (v/v) Triton X-100 and measured by microphotometry at 550 nm.

2.4. Cell cycle analysis by flow cytometry

MCF-7 cells seeded in Petri dishes were treated with E_2 at 0.1 nM or ERα17p at 10 μM for 6, 24 and 48 h. Subconfluent cells were then trypsinized, washed twice with PBS and stained using Coulter DNAPrep reagent kit (Beckman Coulter; Miami, FL). Briefly, cells were suspended in 50 μl of reagent A (15 s, 20 °C, under vortex agitation) and incubated in staining solution (950 μl of reagent B). After incubation (2 h, 4 °C, dark), cell cycle distribution was analyzed with a BD FACSCalibur analyzer (BD Biosciences; Erembodegem, Belgium) and WinCycle software (Phoenix Flow Systems; San Diego, CA).

2.5. Western blot analysis

After 24 h of treatment with E_2 at 0.1 nM, ERα17p at 10 μM and/or OH-Tam at 0.1 μM, MCF-7 cells were washed with PBS before lysis in TBS (50 mM Tris–HCl pH 7.5 with 150 mM NaCl) containing 1% IGEPAL, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM NaF, 0.1 mM sodium orthovanadate, 5 mM EDTA, 0.6 mM PMSF and 0.3 mM TPCK. Lysates were clarified and protein concentration of each sample was determined by BCA protein assay kit (Pierce). After addition of LDS Sample 4X buffer (Invitrogen), samples were boiled for 5 min and submitted to Western blot analysis as previously described [30]. Immunodetection was performed by chemiluminescence, using a peroxidase-coupled secondary antibody and Western Pico Detection system (Pierce). Immunoblots were visualized using FLA-3000 camera (Fuji; Tokyo, Japan).

2.6. ERα mRNA assessment by nucleic acid sequence based amplification (NASBA)

MCF-7 cells were exposed to E_2 at 0.1 nM or ERα17p at 10 μM for 6, 24 and 48 h. Cells were then harvested and their RNAs were extracted with TriPure (Roche Applied Science) for NASBA analysis [45,46] (kindly performed by Thibault Verjat and Bruno Mougin from BioMérieux; Lyon, France). Briefly, 5 ng of RNA were added to 10 μl of NASBA buffer (final concentrations in 20 μl reaction mixture: 40 mM Tris HCl, pH 8.5, 12 mM $MgCl_2$, 70 mM KCl, 5 mM dithiothreitol, 15% (v/v) DMSO, 1 mM dNTP, 2 mM NTP, 0.2 μM of ERα and cyclophilin B (PPIB) primers, and 0.1 μM of specific ERα and PPIB molecular beacons). Samples were preincubated successively at 65 °C and 41 °C (2 min each). Five microlitres of enzyme mix (0.08 U RNase H, 32 U T7-RNA polymerase, 6.4 U reverse transcriptase, RT) were then added for a further incubation at 41 °C for 90 min. ERα mRNA levels were normalized with respect to PPIB mRNA levels.

2.7. Assessment of ERα turnover by [^{35}S]methionine labeling

2.7.1. ERα synthesis

Medium of MCF-7 subconfluent cultures was removed and replaced by EMEM devoid of L-methionine. After incubation (2 h, 37 °C) cells were exposed to 10 nM L-[^{35}S]methionine (2 h, 37 °C). Cells were lysed as described above, lysates were clarified and protein concentrations were determined by BCA protein assay kit (Pierce). Lysates were immunoprecipitated as previously described [47]. Briefly, samples containing 250 μg of total protein were precleared using agarose-coupled anti-rabbit IgG (45 μl; 50% slurry, 2 h, 4 °C). Precleared lysates were incubated with HC-20 anti-ERα antibody (2 μg, overnight, 4 °C). ERα-antibody complexes were precipitated by an agarose coupled anti-rabbit IgG (45 μl; 50% slurry, 2 h, 4 °C) and pellets were washed four times with 900 μl of lysis buffer. Pellets were then suspended in 60 μl LDS 1×, boiled for 5 min. Twenty-five microlitres of denatured samples were submitted to electrophoresis (12% SDS-PAGE; Invitrogen). Gels were fixed (10% acetic acid, 40% methanol, 50% ddH_2O; 30 min, room temperature), washed with ddH_2O (30 min, room temperature) and incubated (90 min, room temperature) in presence of salicylic acid (1 M salicylic acid, 40% ethanol in ddH_2O) in order to enhance radioactive signals. Finally gels were dried and submitted to fluorography to detect radiolabeled ERα bands (67 kDa) (3 days, −80 °C) with hyperfilms MP (GE Healthcare). Densitometric analyses were performed using a GS-710 Calibrated Imaging densitometer and Quantity One software (BioRad; Hercules, CA)

2.7.2. ERα degradation

After labeling (as described above), cells were allowed to grow in fresh medium (without [^{35}S]methionine) for 0, 1, 2, 3 or 4 h before harvesting (chase experiment). Non degraded [^{35}S]-labeled ERα was quantified as described above.

2.8. Pull-down experiments

ERα17p-Biotin was linked to streptavidin–agarose beads in TBS containing 1% IGEPAL and 1 mg/ml of BSA (2 h, room temperature). Excess of ERα17p-Biotin was then removed by several washes with TBS containing 1% IGEPAL. Subconfluent cultures of MCF-7 cells were lysed in TBS containing 1% IGEPAL, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM NaF, 0.1 mM orthovanadate, 0.6 mM PMSF, 0.3 mM TPCK and a cocktail of protease inhibitors (Complete Mini; Roche). Lysates were clarified and protein concentrations were determined as described above. Extracts (500 μg of proteins in ∼1 ml) were precleared using unlinked streptavidin–agarose beads (100 μl, 50% slurry; 2 h, 4 °C). ERα17p-interacting proteins were then recovered using ERα17p-linked beads (100 μl, 50% slurry; 2 h,

4 °C); unlinked streptavidin–agarose beads were used in parallel for assessing potential non-specific binding (NS). ERα17p at 10 μM was also added to the mixture as an additional control for binding specificity. After washing, beads were suspended in 50 μl of LDS 1X electrophoresis buffer and boiled for 5 min. Samples were then submitted to SDS-PAGE and the proteins were revealed by Coomassie staining. Additionally, proteins of interest were revealed by Western blotting as described above.

2.9. Protein digestion and analysis by automated liquid chromatography–tandem mass spectrometry (LC–MS/MS)

Coomassie-stained protein bands were manually excised for in-gel-digestion. Briefly, proteins were in-gel reduced by dithiothreitol (DTT) at 56 °C for 30 min and alkylated with iodoacetamide (dark cabinet, 20 min, room temperature) before proteolytic digestion with bovine trypsin (Roche) for 2 h at 37 °C, according to the Shevchenko protocol [48]. After acidification of the tryptic digest by addition of 1 vol. of 5% aqueous formic acid (FA), proteolytic peptides were analyzed by nanoscale capillary liquid chromatography–tandem mass spectrometry (LC–MS/MS): a capillary LC system (Famos-Switchos-UltiMate Dionex, LC Packings; Amsterdam, The Netherlands) was used online with a hybrid nanoESI quadrupole-time of flight mass spectrometer (Q-TOF2, Waters, Micromass; Manchester, UK). After an on-line desalting and concentration on a precolumn (Pepmap C18, 0.3 mm i.d., 5 mm, Dionex), chromatographic separations were conducted on a reverse-phase (RP) capillary column (Atlantis dC18 Nanoease™ column 3 μm, 75 μm i.d., 15 cm length, Waters; Milford, MA) at a flow rate of 200 nl/min. Samples of 2.5 μl were diluted in 6 μl of buffer A (H_2O/acetonitrile/FA, 96/4/0.1, v/v). The gradient profile used consisted in a desalting step at 100% A for 6 min, and a linear gradient from 0% B to 30% B (H_2O/acetonitrile/FA, 10/90/0.085, v/v) in 9 min, 30% B to 60% B in 35 min, followed by a flush at 100% B over 15 min. Data acquisition was achieved in automatic mode switching between the survey acquisition in MS and fragmentation acquisition in MS/MS on the four most intense ions detected in the former survey scan. Mass data collected during an LC–MS/MS analysis were processed and converted into a .PKL file using the Masslynx software (Micromass) before being submitted to the search software Mascot (http://www.matrixscience.com/). Protein identifications were obtained by comparison of experimental data with the Swissprot database (Release 53.2 of 26 June 2007) with no restriction on taxonomy, 2 miscleavages, 0.3 Da tolerance on MS data, 0.5 Da tolerance on MS/MS data, and variable oxidation on Met and carbamidomethylation on Cys.

2.10. Co-immunoprecipitation

Subconfluent MCF-7 cells seeded in T-175 flasks were incubated for 20 min with E_2 at 0.1 nM or with ERα17p at 10 μM. They were then washed twice with PBS, harvested by scraping and lysed in TBS containing 0.5% IGEPAL, 0.5 M KCl, 0.6 mM PMSF and 0.3 mM TPCK. After dilution (4×, in order to decrease KCl concentration), lysates were clarified by centrifugation. Samples were either directly submitted to Western blotting (inputs; detection of ERα and Hsp70) or submitted to ERα immunoprecipitation as described above prior Western blotting (using F-10 primary mouse antibody and agarose-coupled anti-mouse antibody). Amounts of ERα and Hsp70 were evaluated by Western blotting. Densitometric analyses were performed using Aida software.

2.11. Statistical analyses

Statistical analyses were performed by ANOVA, followed by post-hoc Tukey's test using SPSS software (Paris, France).

3. Results

3.1. ERα17p stimulates MCF-7 cell proliferation

The ability of ERα17p to increase the proliferation of ERα-positive breast cancer cell lines in steroid-depleted medium

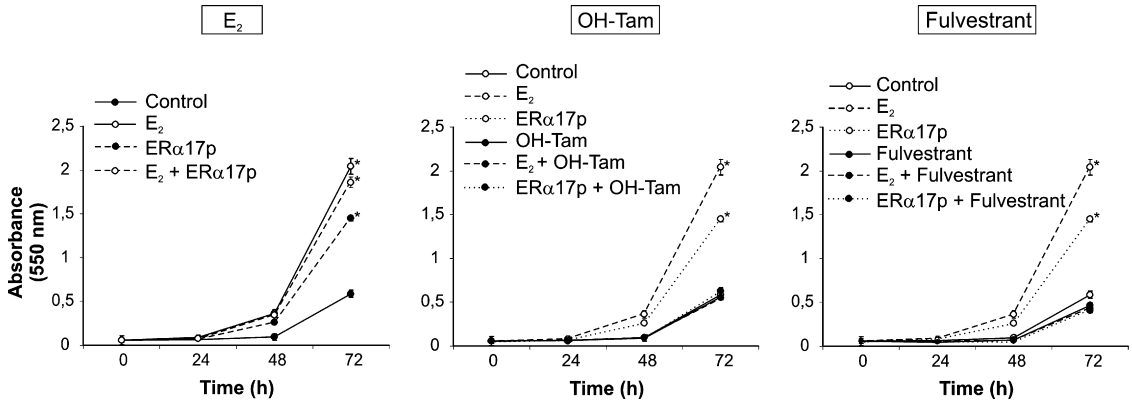


Fig. 1. Modulation of ERα17p-induced MCF-7 cell growth stimulation by E_2, OH-Tam and fulvestrant. MCF-7 cells were grown for 24, 48 and 72 h in the absence (control) or in the presence of ERα17p at 10 μM with or without E_2 (0.1 nM), OH-Tam or Fulvestrant (both at 1 μM). Cell growth was measured by crystal violet staining. Measurements were performed in sixplicate. Data refer to the mean value ± S.D. of a representative experiment performed three times. *, Significantly different ($p < 0.001$) from control after 72 h of treatment.

(DCC-treated serum) has been reported in a previous study [30]. The fact that the peptide was unable to exhibit a similar property on ERα-negative cells indicated the implication of ERα-dependent pathway(s) in its mode of action. As a further confirmation, we show here (Fig. 1) that the trophic effects of ERα17p and E_2 on MCF-7 cells are not additive, indicating that they affect the same pharmacological target (i.e. the receptor). In agreement with this view, antiestrogens (i.e. OH-Tam and fulvestrant) abrogate the stimulation of growth induced by ERα17p.

To understand how ERα17p operates, we assessed its effect on MCF-7 cell cycle distribution. Fig. 2A shows that after 24 and 48 h of treatment, ERα17p, like E_2, increases cell frequency in both S and G2/M phases. No change was recorded after 6 h. When presented as bar plots (Fig. 2B), our data reveal that ERα17p and E_2 maintained cell frequencies in S and G2/M initial levels while in control conditions cells tended to accumulate in G0/G1 phases. Thus, ERα17p seems able to compensate for the effect of estrogen depletion and/or serum starvation, acting as a substitute for E_2.

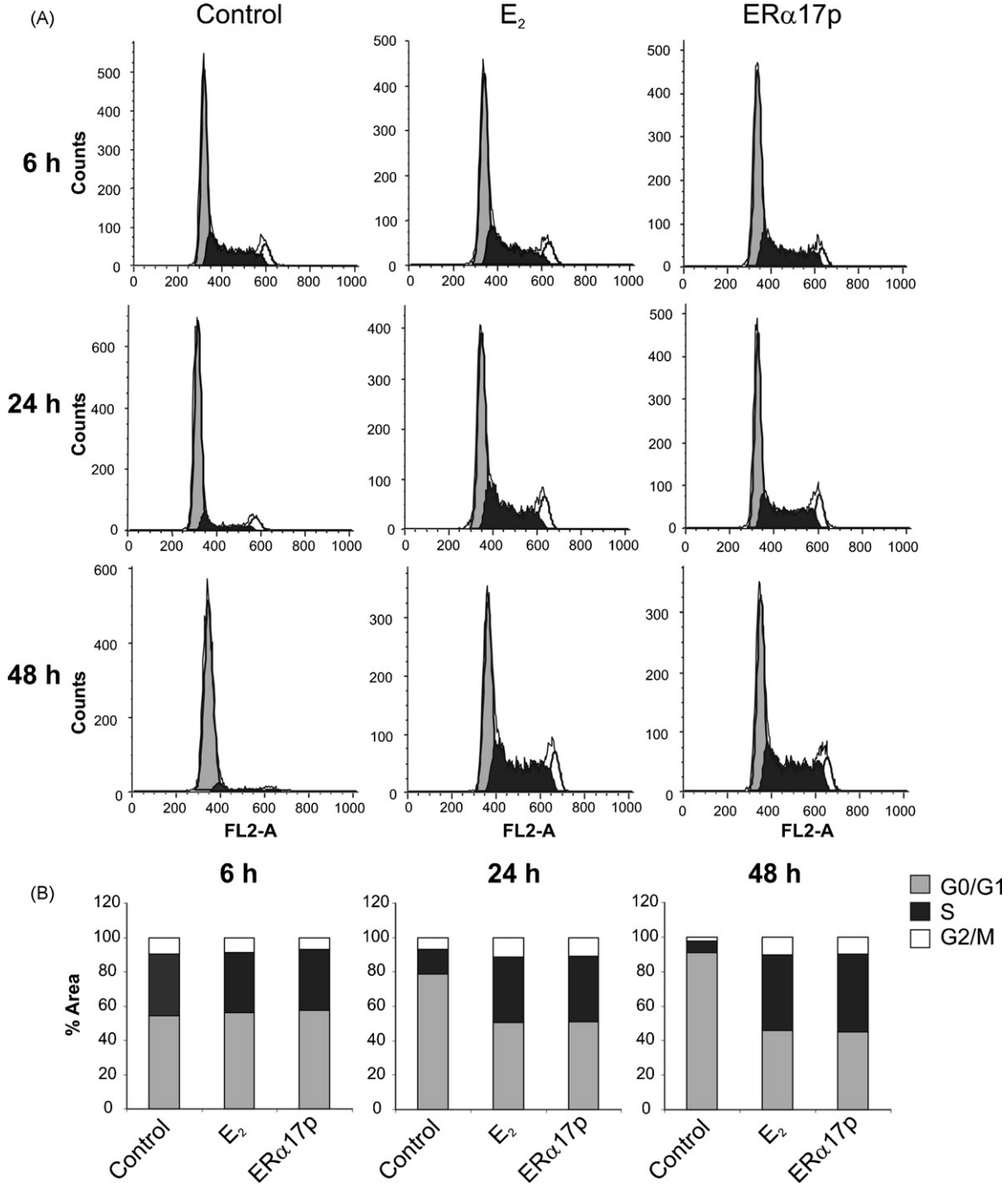


Fig. 2. Effect of ERα17p on cell cycle phase distribution. MCF-7 cells were incubated for 6, 24 and 48 h in the absence (control) or presence of E_2 at 0.1 nM or ERα17p at 10 μM. (A) Representative data from flow cytometry analyses. (B) Bar plots giving the percentages of cells in G0/G1, S, and G2/M, as estimated from the areas under the curves.

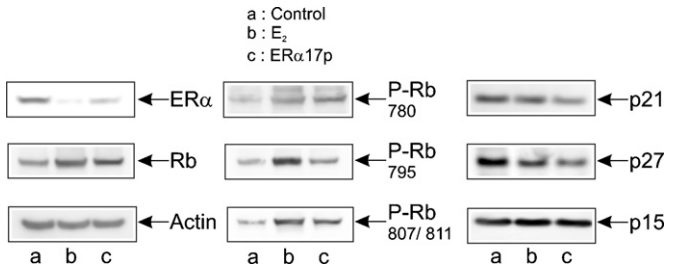


Fig. 3. Influence of ERα17p on the G1/S cell cycle transition checkpoint. MCF-7 cells were incubated in the absence (control; a) or presence of E_2 at 0.1 nM (b) or ERα17p at 10 μM (c). Amounts of ERα, Rb, Actin, Rb phosphorylated at Ser780, 795 and 807/811, p21, p27 and p15 were assayed by Western blot analysis using appropriate primary and secondary antibodies. Immunoblots are representative of, at least, two independent experiments.

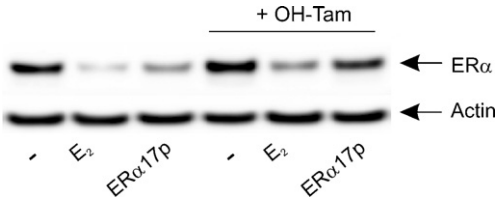


Fig. 4. Influence of OH-Tam on ERα17p-induced ERα down regulation. MCF-7 cells were incubated in the absence (control) or presence of E_2 at 0.1 nM or ERα17p at 10 μM with or without OH-Tam at 0.1 μM. ERα and actin levels were then assayed by Western blot analysis using F-10 and MAB1501R antibodies. Immunoblots are representative of three independent experiments.

Results from cell kinetic analysis were corroborated by the demonstration of enhanced phosphorylation of the retinoblastoma protein (Rb; G1/S transition checkpoint) after 24 h of treatment (Fig. 3). ERα17p and E_2 similarly increased Ser780 phosphorylation, while ERα17p was less effective than E_2 in inducing Ser795 phosphorylation. This should not affect entrance of the cells into S phase since phosphorylation of Ser780 is sufficient to induce the required release of E2F [49].

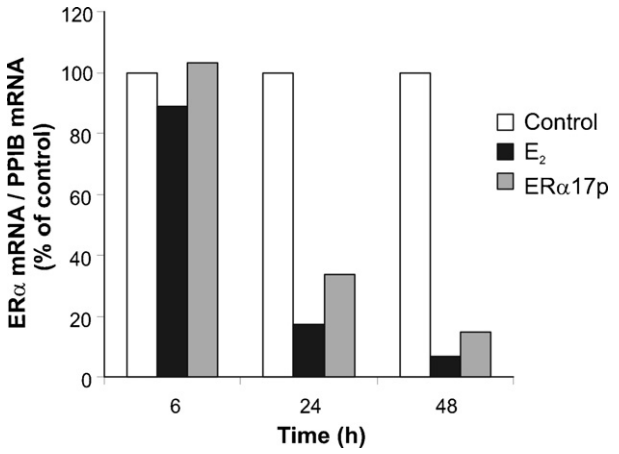


Fig. 5. Influence of ERα17p on ERα mRNA level. MCF-7 cells were cultured in absence or presence of E_2 at 1 nM or ERα17p at 10 μM for 6, 24 and 48 h. ERα mRNA level was measured by NASBA and normalized according to cyclophilin B (PPIB) mRNA. Figure refers to the means of two independent experiments, which gave similar results (variation around the mean did not exceed 5%).

Phosphorylation of Ser807/811, which promotes c-Abl release, was also recorded with both ERα17p and E_2. All these changes were correlated with a diminution of p21, the kinase inhibitor which suppresses Rb phosphorylation by Cdk 2–Cyclin A/E and Cdk 4–Cyclin D1/D2 complexes. In a similar way, the kinase inhibitor p27, which is normally induced by serum starvation and abrogates the activity of Cdk 2–Cyclin A/E complexes, was also found to decrease. Of note, no decrease of the Cdk 4–Cyclin D1 complex inhibitor p15 was recorded.

3.2. ERα17p induces ERα down regulation

Data presented in Fig. 3 show that ERα17p-induced events related to enhanced cell proliferation are associated with a loss of ERα. As depicted in Fig. 4, this loss was inhibited by OH-

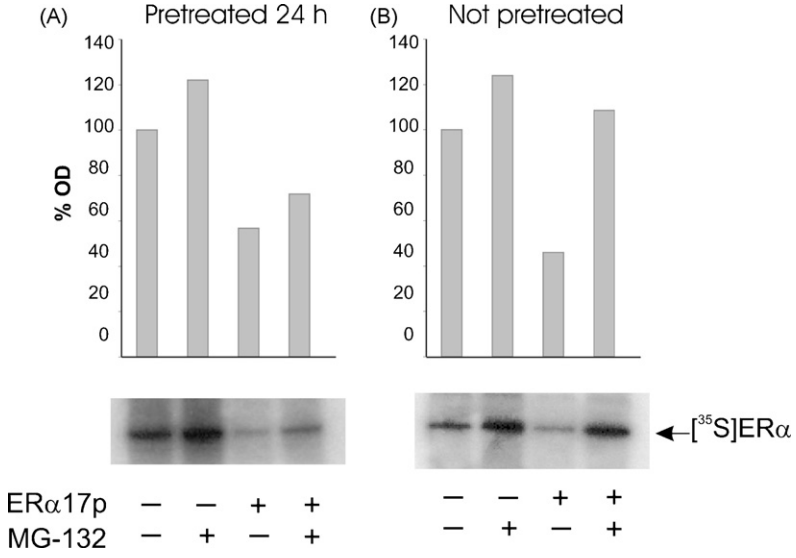


Fig. 6. Influence of ERα17p on ERα synthesis. MCF-7 cells were pretreated (A) or not (B) for 24 h with ERα17p at 10 μM. Cells were then exposed to 10 nM [^{35}S]methionine for 2 h with or without ERα17p and/or MG-132 both at 10 μM. Immunoprecipitated [^{35}S]ERα from cell extracts were then submitted to SDS-PAGE, revealed by autofluorography, and quantified by measuring band intensities. Columns represent mean intensities calculated from two independent experiments.

Tam. This finding is not surprising since in most breast cancer cell lines mitogenic response to estrogens is usually associated with a down regulation of ERα [41,42,50], resulting from a rapid increase of its degradation rate followed by a repression of its gene (see Ref. [51] for review). Of note, ERα absence recorded in breast tumors *in vivo* could also be the consequence of these two phenomena: in addition to the well-known ERα gene silencing, receptor half-life has also been shown to decrease under some circumstances [52].

In order to assess whether ERα17p acts like a *bona fide* estrogen agonist, we analyzed its effect on ESR1 gene transcription. We found that ERα17p, like E_2, drastically decreases ERα mRNA level after 24 and 48 h of treatment (Fig. 5), clearly indicating that it represses ERα gene transcription. This property was confirmed by exposure of the cells to [^{35}S]methionine after a pretreatment of 24 h with ERα17p (Fig. 6A). Indeed, concomitantly with a decline of ERα mRNA level, we recorded a decrease of neosynthesized ERα (identified by [^{35}S] labeling; lane 1 vs. lane 3). This decrease could not be ascribed to enhanced proteasomal degradation since it was not reversed by MG-132 (lane 4). By contrast, when ERα17p was added to cells at the time of [^{35}S]methionine incorporation (Fig. 6B; no pretreatment), we also observed a decrease of [^{35}S]ERα, but the latter was abrogated by MG-132 (lane 3 vs. lane 4), suggesting a degradation of neosynthesized receptor via the ubiquitin–proteasome pathway.

Pulse-chase experiments confirmed the ability of ERα17p to enhance the rate of receptor degradation. Thus, when MCF-7 cells were treated with ERα17p after [^{35}S] labeling (Fig. 7A), we observed a more rapid decline of [^{35}S]ERα (decreased half-live), like what was seen with E_2. Interestingly, OH-Tam abrogated the enhancing effect of ERα17p on ERα degradation while it was ineffective in the case of E_2 (Fig. 7B). That could be due to the fact that ERα17p does not interact with the receptor at the ligand-binding site and therefore cannot compete with OH-Tam. In agreement with this statement, we found that ERα17p does not modify the relative binding affinity of OH-Tam for a purified recombinant hERα in a classical [^{3}H]E_2 binding assay (data not shown). On the other hand, cycloheximide also suppressed the ERα17p-induced degradation of the receptor (Fig. 7C), a property shown to only occur under estrogenic stimulation [53–55].

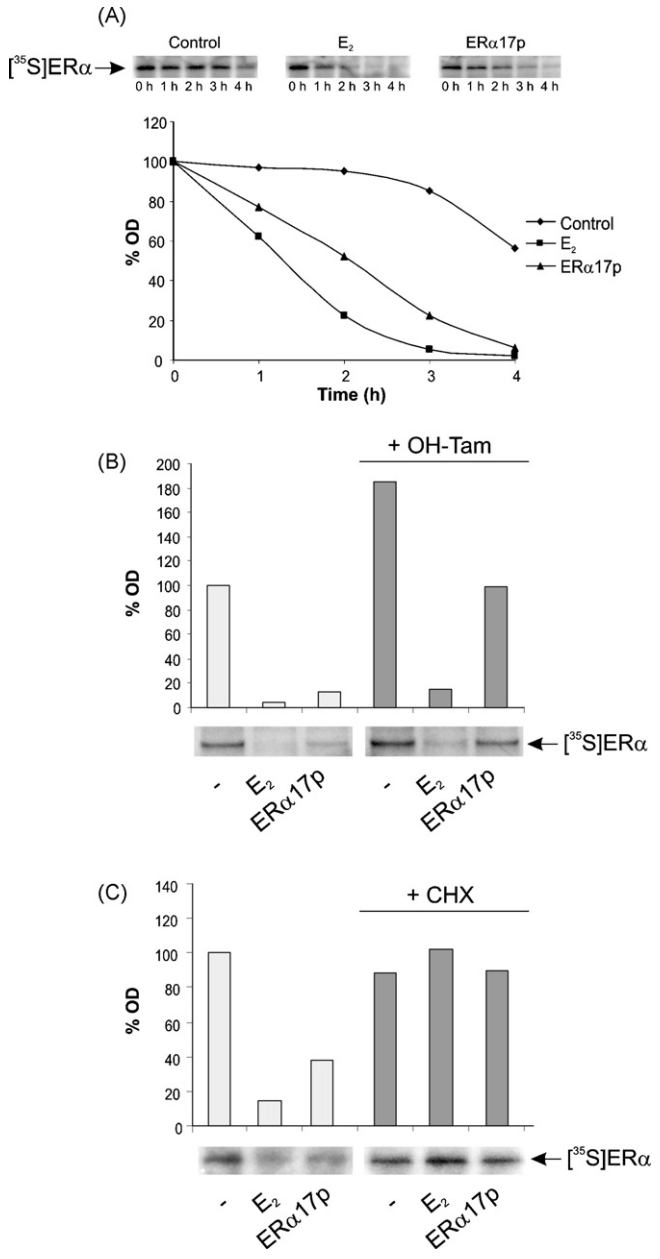
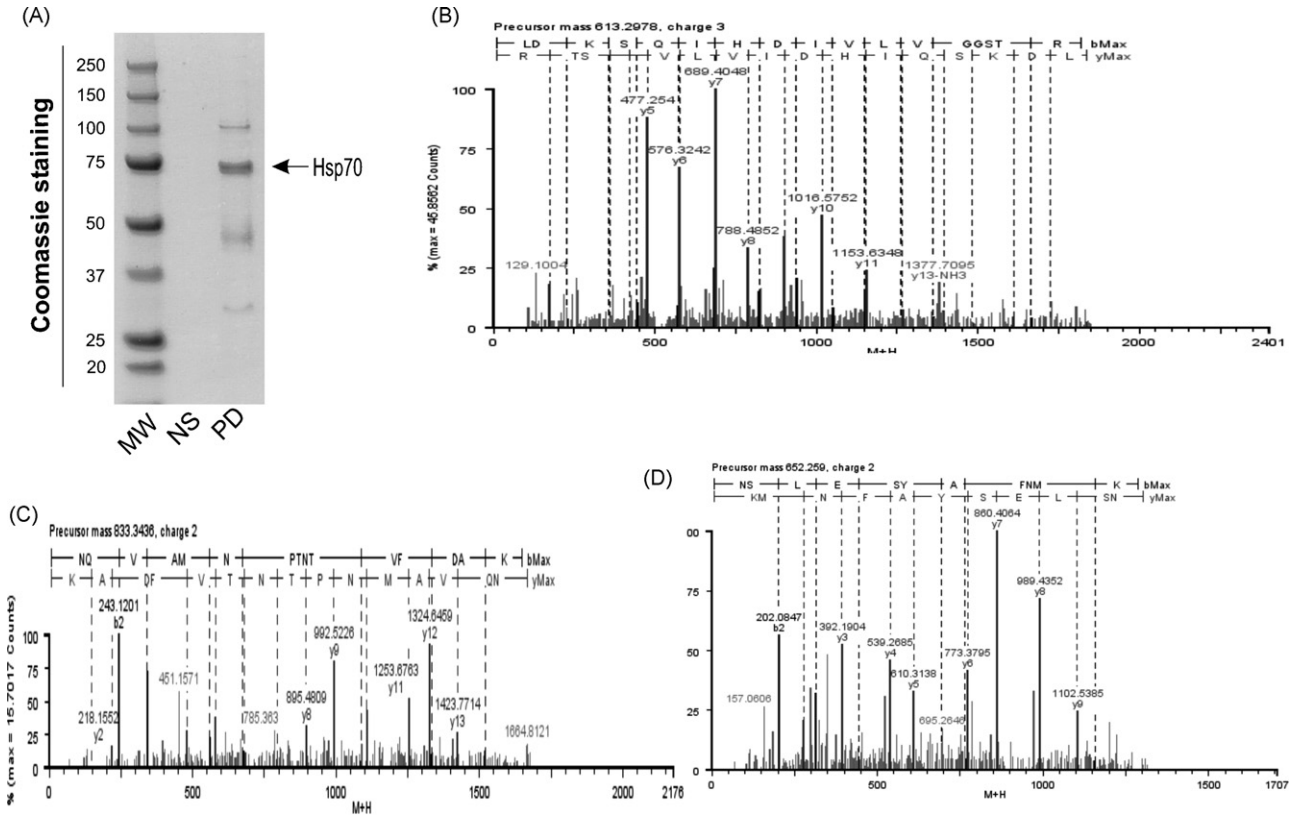


Fig. 7. Effect of ERα17p on ERα degradation. MCF-7 cells were labeled with 10 nM [^{35}S]methionine for 2 h. After removal of the medium, cells were maintained in culture without (control) or with (A) E_2 at 1 nM or ERα17p at 10 μM for 1–4 h; (B) E_2 at 1 nM or ERα17p at 10 μM in the absence or presence of OH-Tam at 0.1 μM for 4 h; (C) E_2 at 1 nM or ERα17p at 10 μM in the absence or presence of cycloheximide (CHX) at 50 μM for 4 h. Immunoprecipitated [^{35}S]ERα from cell extracts was then submitted to SDS-PAGE, revealed by autofluorography, and quantified by measuring band intensities. Plot and columns represent mean intensities calculated from two independent experiments.

3.3. Hsp70 is involved in ERα17p mechanism of action

Proteasomal-dependent ERα degradation illustrated above is a process requiring association and/or dissociation of coregulators. Search for such potential coregulator(s) was carried out by pull-down experiments on MCF-7 cells extracts using ERα17p-linked beads. Only a few proteins were isolated by this procedure (Fig. 8A), suggesting that endogenous candidates for association with the P_{295}-T_{311} motif are rather scarce. YB-1 (Y box-binding protein 1) and 70-kDa heat shock family members were identified by LC–MS/MS analysis (Fig. 8B–E). The presence of Hsp70-1, for which a high identification score was recorded, was confirmed by Western blot analysis (Fig. 8F); for unknown reason the same immunoblotting procedure failed to confirm YB-1 (data not shown). On the other hand, we looked for similar association of ERα17p with ERα and CaM since we had previously shown that the peptide associates with a purified preparation of recombinant ERα [36] as well as with CaM immobilized onto a Sepharose matrix [30]. No significant signals for both proteins were recorded in our pull-down experiments (data not shown) suggesting that, in a cellular environment, ERα17p would preferentially interact with Hsp70. This behavior may result either from a greater affinity for Hsp70 or from the fact



(E)

Mw (kD)	access N°	Protein	Theor. Mw (kD)	Identified peptide	Coverage (%)
~ 100		No identification with high confidence			
70-75	HSP7C_HUMAN	Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8) - Homo sapiens	70,854	8	12
	HSP71_HUMAN	Heat shock 70 kDa protein 1 (HSP70.1) (HSP70-1/HSP70-2) - Homo sapiens	69,995	8	15
	GRP78_HUMAN	78 kDa glucose-regulated protein precursor (GRP 78) (Heat shock 70 kDa protein 5) (Immunoglobulin heavy chain-binding protein) (BiP) (Endoplasmic reticulum lumenal Ca(2+)-binding protein grp78) - Homo sapiens	72,288	9	20
	GRP75_HUMAN	Stress-70 protein, mitochondrial precursor (75 kDa glucose-regulated protein) (GRP 75) (Peptide-binding protein 74) (PBP74) (Mortalin) (MOT) - Homo sapiens	73,635	4	7
45-50	YBOX1_HUMAN	Nuclease sensitive element-binding protein 1 (Y-box-binding protein 1) (Y-box transcription factor) (YB-1) (CCAAT-binding transcription factor I subunit A) (CBF-A) (Enhancer factor I subunit A) (EFI-A) (DNA-binding protein B) (DBPB) - Homo sapiens	35.903	3	17
~ 30		No identification with high confidence			

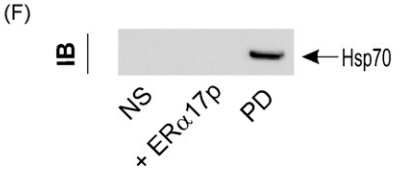


Fig. 8. Identification of Hsp70 as an ERα17p-interacting protein. (A) Pull-down. MCF-7 cells extracts were incubated with streptavidin–agarose beads (non-specific; NS) or ERα17p-linked agarose beads (Pull-down; PD). Extracted proteins were then submitted to SDS-PAGE and revealed by Coomassie staining. (B–D) Experimental MS/MS spectra contributing to the identification of Hsp70 obtained after the fragmentation of, respectively, a triplecharged tryptic peptide of 613.30 Da, a doublecharged tryptic peptide of 833.34 Da and a doublecharged tryptic peptide of 652.26 Da. (E) Table List of identified proteins with their relative mass. (F) Confirmation of the ERα17p–Hsp70 association. Protein extracted with streptavidin–agarose beads (NS), ERα17p-linked agarose beads in the absence (PD) or presence of an excess of free ERα17p (+ERα17p) were submitted to Western blotting using anti-Hsp70 primary antibody.

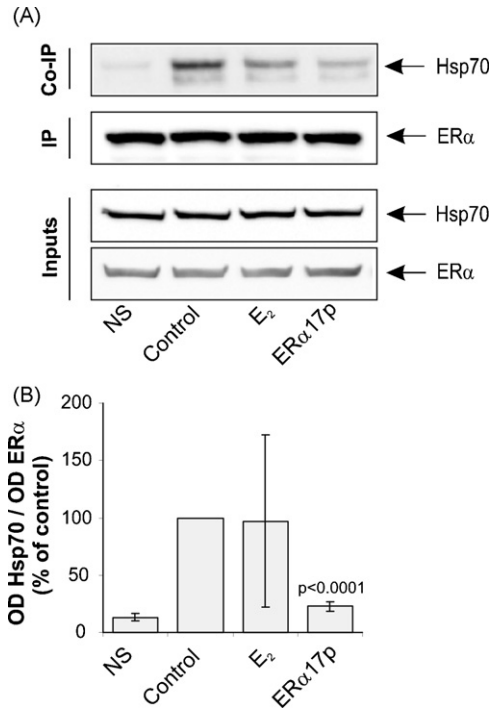


Fig. 9. Inhibition of endogenous ERα–Hsp70 association by ERα17p. MCF-7 cells were treated for 20 min without or with E_2 at 0.1 nM or ERα17p at 10 μM. After cell lysis, ERα–Hsp70 complexes were co-immunoprecipitated with F-10 anti-ERα antibody. Non-specific binding (NS) was assayed by omitting primary antibody. Input and immunoprecipitated levels of ERα and Hsp70 were revealed by Western blotting using HC-20 anti ERα and anti Hsp70 primary antibodies. (A) Figure showing representative immunoblots from four independent experiments. (B) Band intensity analysis of nine independent experiments (mean ratio of Hsp70/ERα intensities).

that the latter protein is in large amount, sufficient to saturate the ERα17p-linked beads.

Complementary ERα–Hsp70 coimmunoprecipitation experiments ($n = 9$) revealed that pretreatment of MCF7 cells with ERα17p produces a rapid and reproducible (20 min; $p < 0.0001$) dissociation of intracellular ERα–Hsp70 complexes (Fig. 9). Interestingly, exposure of cells to E_2 produced erratic effects: while the hormone similarly dissociated ERα–Hsp70 complexes in some experiments (i.e. Fig. 9A), it failed to do so in others (Fig. 9B). This peculiar behavior could be explained by the fact that the release of Hsp70 form ERα-containing oligomeric structures is not a direct consequence of E_2 binding to the receptor but rather a consequence of conformational changes in which environment (i.e. coregulators) plays a critical role. In addition, it should be stressed that Hsp70 associates with ERα at a stage preceding the final maturation step (i.e. recruitment of Hsp90 and immunophilins) which confers to the receptor an optimal conformation for ligand-binding (see Refs. [56,57] for reviews).

Overall, our data suggest that ERα17p, by disrupting ERα–Hsp70 association promotes receptor activation and commitment to proteasomal degradation. This is in agreement with the recently proposed concept that 70 kDa heat shock proteins maintain the unliganded receptor in its inactive status by inhibiting recruitment of coactivators [58].

4. Discussion

In this study, we demonstrate that ERα17p, a synthetic peptide corresponding to the P_{295}-T_{311} sequence in ERα, exerts on MCF-7 cells proliferation an agonistic activity which can be abrogated by cotreatment with two anti-estrogens (OH-Tam and fulvestrant). Analysis of cell cycle and G1/S checkpoint (Rb phosphorylation) reveals that the mitogenic effect of ERα17p corresponds to a relief of cell cycle arrest associated with estrogen deprivation. In agreement with the concept of an inverse relationship between breast cancer cell proliferation and ERα level [50], we show that ERα17p down regulates the receptor by accelerating its degradation (rapid effect) as well as by decreasing the expression of its gene (delayed effect). Interestingly we show here that ERα17p associates with 70-kDa heat shock protein family members, a behavior consistent with its primary and secondary structures [36]. It is, indeed, known that peptides like ERα17p containing hydrophobic and positively charged aminoacids [59] and adopting non-structured or weakly helicoidal conformations [36,60] are targeted by such chaperones. The additional finding that ERα17p inhibits ERα–Hsp70 association in MCF-7 cells could explain why it induces ERα degradation, inasmuch as heat shock proteins are known to contribute to the stability of the receptor.

Distinct ubiquitin–proteasome systems (UPS) seem to contribute to basal and ligand-induced ERα degradation. The finding that the proteasome inhibitor MG-132 inhibits ERα17p-induced ERα degradation demonstrates the involvement of an UPS pathway in this elimination process. Interestingly, when the receptor is unliganded, CHIP (carboxyl terminus of Hsp70-interacting protein) has been shown to mediate a cytoplasmic ERα ubiquitination/degradation which is not associated with enhanced ERE-dependent transcription [61,62]. Since ERα17p-induced ERα elimination is accompanied by ERα-mediated gene transcription, one may surmise that it occurs independently of CHIP. It might thus involve another ubiquitin ligase system associated with ERα activation and subsequent gene transcription [30,36]. In support of this view, siRNA raised against CHIP has been shown to enhance the basal transcription of pS2, a well-known ERE-driven, ERα target gene [61]. Among ubiquitin ligases that could be responsible of the ERα17p-induced ERα degradation/activation, E6-AP is a plausible candidate since it ubiquitinates ERα by a mechanism that requires the release of calmodulin [32] from its binding site onto the receptor, i.e. the P_{295}-T_{311} motif [30]. Moreover, this ubiquitin ligase is involved in both the hormone-dependent degradation and the transcriptional activity of the progesterone and androgen receptors [63,64]. However, involvement of other ubiquitin ligases such as MDM2 or EFP (estrogen-responsive finger protein) could not be excluded since they have also been described to mediate agonist-induced degradation of ERα [65,66].

Our pull-down experiments reveal that 70-kDa heat shock protein family members associate with ERα17p, suggesting that these chaperones may interact with the P_{295}-T_{311} motif of the receptor. Interestingly, Hsp70-8 (Hsc70) has also been reported to associate with ERα [58], via the M_{396}-F_{461} region located in the spatial vicinity of the P_{295}-T_{311} motif. One may therefore

suppose that 70-kDa heat shock protein-containing complexes could link various regions of the receptor, maintaining it in a stable and inactive conformation. Interestingly, deletions within the P_{295}-T_{311} motif generate unstable receptor mutants exhibiting high basal transcriptional activity (Refs. [29,30] and unpublished data). Accordingly, we propose that mutations located within this motif (i.e. K303R [67]) may have an impact on Hsp70 association with concomitant modification of the receptor activation/degradation cycles. Such considerations may be extended to orphan estrogen receptors (estrogen related receptors; ERRs) to explain their known constitutive transcriptional activity. Indeed, according to sequence alignments, we found that ERRs, and especially ERRβ and γ, display a gap in the P_{295}-T_{311} ERα-motif (N_{304}SLALSL$_{310}$ corresponding region). We, thus, anticipate that this gap may affect 70-kDa heat shock protein binding to ERRs giving rise to CHIP-independent basal degradation and high basal transcription. Study of ERR turnover as well as 70-KDa heat shock protein association would confirm or invalidate this hypothesis.

On another point of view, it is established that ERα ligands produce a rapid decrease of its capacity to bind [^{3}H]E_2 in MCF-7 cells. As shown by previous time-course studies, this loss of binding capacity cannot be ascribed to ERα degradation [68,69]. We recently reported that several molecules unable to compete with labeled hormone for receptor binding produce a similar effect on ERα, indicating that it is independent of interactions with residues involved in the recognition of E_2 [70]. Of note, we previously reported that ERα17p similarly alters ERα binding capacity, while it fails to compete with [^{3}H]E_2 for binding to purified human ERα in a cell-free system [36]. This peculiar phenomenon that could be ascribed to an early conformational change may find its origin in the dissociation of Hsp70-stabilized oligomeric structures containing ERα. Hence, according to this view, all molecules able to produce such dissociation would liberate ERα and promote receptor-induced transactivation.

Globally, our data suggest that ERα17p, via its ability to dissociate ERα–Hsp70 complexes, may relax the receptor (conformational changes associated with a loss of [^{3}H]E_2 binding capacity) leading to its progressive elimination, an initial step in a complex mechanism eventually resulting in cell growth [30,36]. Hence, we assume that the design of drugs that would stabilize Hsp70-induced inactive ERα conformation may be of therapeutic value for treatment of ERα-related diseases, especially those that are resistant to anti-estrogen therapies.

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