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Calmodulin-independent, agonistic properties of a peptide containing the calmodulin binding site of estrogen receptor α

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Abstract

Calmodulin (CaM) contributes to estrogen receptor α (ER)-mediated transcription. In order to study the underlying mechanisms, we synthesized a peptide including the CaM binding site: ERα17p (P_{295}-T_{311}). This peptide inhibited ER-CaM association, unlike two analogs in which two amino acids required for CaM binding were substituted. Exposure of MCF-7 cells to ERα17p down regulated ER, stimulated ER-dependent transcription and enhanced the proliferation of ER-positive breast cancer cell lines. Interestingly, ERα17p analogs unable to bind to CaM induced similar responses, demonstrating that ERα17p-mediated effects are mainly relevant to mechanisms independent of ER-CaM dissociation. The P_{295}-T_{311} motif is indeed a platform for multiple post-translational modifications not necessarily CaM-dependent. The additional finding that deletion of the P_{295}-T_{311} sequence in ER produced a constitutive transcriptional activity revealed that this platform motif has autorepressive functions. With regard to cell function, association of CaM to ER would counteract this autorepression, leading thereby to enhanced ER-mediated transactivation.
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1. Introduction

Implication of estrogens and their receptors in growth, differentiation and function of a variety of target tissues is now well established. Two estrogen receptor isoforms (α and β) have been identified, estrogen receptor α (hereinafter referred to as ER) is the dominant isoform expressed in breast cancer. Studies of our laboratory are devoted to the analysis of the mechanisms by which this receptor contributes to the pathogenesis of breast tumors. ER belongs to the superfamily of nuclear receptors which are known to act as ligand-dependent transcription factors (Evans, 1988; Leclercq et al., 2006). Actually, ER biological activities are modulated by formation of diverse transient complexes with a variety of co-regulators (Rossini, 1994). Calmodulin (CaM), an ubiquitous Ca^{++} sensor protein, is one of these co-regulators, the importance of which in ER-mediated transcription is now well recognized (Biswas et al., 1998; Garcia Pedrero et al., 2002; Li et al., 2003; Li et al., 2005).

Early studies revealed that the association of CaM with ER enhances the binding of the latter to estrogen response elements (ERE) (Biswas et al., 1998; Bouhoute and Leclercq, 1995), a step of prime importance for initiating the transcription of estrogen-regulated genes. There is indeed a general consensus that transcription occurs according to a well defined cyclic process involving the combinatorial and sequential

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recruitment of enzymes and factors required for the assembly of transcriptionally productive complexes (Reid et al., 2003; Metivier et al., 2003; Shang et al., 2000). In each cycle, proteasomal degradation of ER molecules that have fulfilled their role in initiation has to occur since the promoters of target genes must be free for the assembly of new complexes. This elimination process facilitates the access of newly synthesized receptors to the promoters, in order to initiate subsequent transcriptional cycles required for maintaining high gene expression. Hence, mechanisms that govern transcription and turnover rate of ER are closely interrelated (Reid et al., 2003; Metivier et al., 2003; Yan et al., 2003; Laios et al., 2005). Unexpectedly CaM, while being an important determinant of ER-mediated transcription (Biswas et al., 1998; Garcia Pedrero et al., 2002; Li et al., 2003, 2005) has been reported to protect ER against proteasomal degradation (Castoria et al., 1988; Li et al., 2001, 2006). This paradox led us to explore further the mechanism of action of this co-regulator.

Current studies aimed at analyzing the mechanism of action of nuclear receptors and their co-regulators often concentrate on the identification of small amino acid sequences putatively involved in specific protein-protein interactions. In this regard, synthetic peptides including such sequences are valuable tools for studying the biological significance of receptor–co-regulator interactions, as well as for the development of selective antagonists (Xu et al., 2002; Iannone et al., 2004; Rodriguez et al., 2004; Shao et al., 2004). It seemed therefore to us that a peptide corresponding to the CaM binding site in ER would prove to be useful for understanding the mechanism by which CaM cooperates with the receptor in the regulation of gene expression. The present paper describes the biological properties of a 17 amino acids synthetic peptide (ERα17p; Fig. 1C) containing an ER motif known to be involved in CaM binding (Castoria et al., 1988; Garcia Pedrero et al., 2002; Li et al., 2005).

2. Material and methods

2.1. Chemicals

Calmodulin-Sepharose 4B and [2,4,6,7-^{3}H]estradiol ([^{3}H]E_2; ∼100 Ci/mmol) were purchased from Amersham Biosciences (Buckinghamshire, U.K.). Highly purified human recombinant ER (hER) and MG-132 were obtained from Calbiochem (San Diego, CA). Estradiol (E_2), calmidazolium chloride, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), ophiobolin A, dansylated CaM, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma (St Louis, MO). Tosyl-L-phenylalaninechloromethylketone (TPCK) came from Roche Diagnostics (Mannheim, Germany).

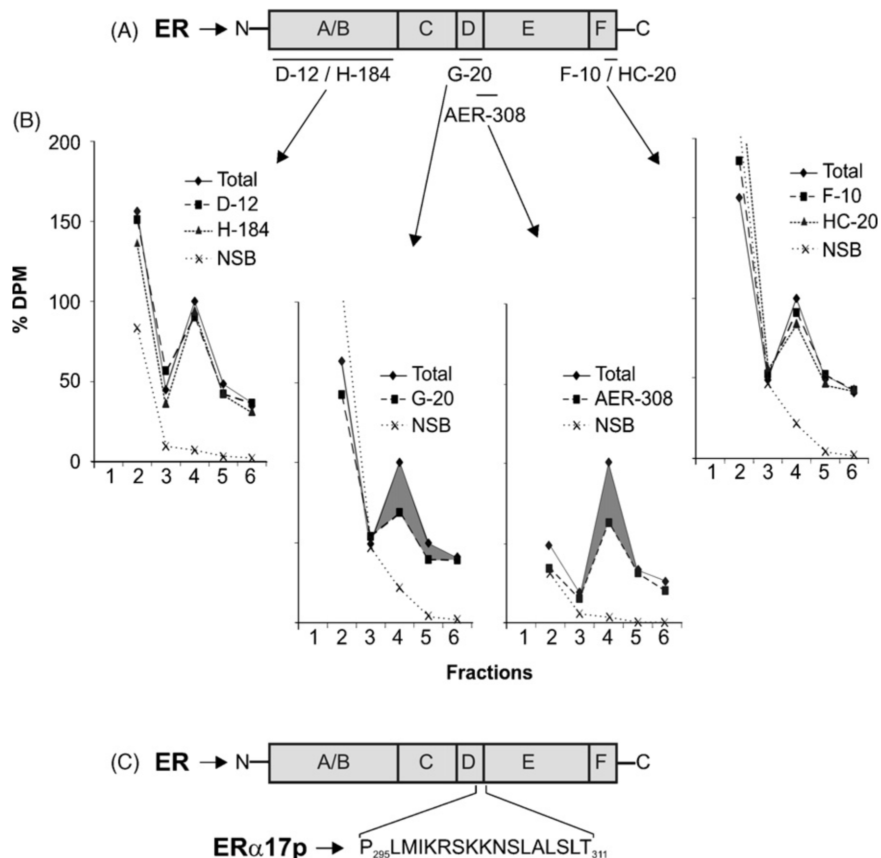


Fig. 1. Influence of anti-ER antibodies on ER-CaM binding. (A) ER regions recognized by D-12, H-184, G-20, AER-308, F-10 and HC-20 anti-ER antibodies. (B) ER–CaM-Sepharose binding assay. [^{3}H]E_2 labeled hER preparations were incubated with CaM-Sepharose in the absence (total binding; ♦) or presence (■ or ▲) of a given anti-ER antibody. Non-specific binding (NSB; ×) was established with a 200-fold excess of unlabeled E_2. After washing (fractions 1–3) matrixes were eluted with EDTA (fractions 4–6) in order to evaluate the potential influence of antibodies on the association of ER to CaM. Grey surfaces represent the decrease of ER-bound CaM. The figure refers to an experiment performed twice. (C) Amino acids sequence of ERα17p and location of the corresponding motif within ER.

2.2. Antibodies

Anti-ER antibodies—i.e. D-12 (mouse monoclonal) and H-184 (rabbit polyclonal) raised against amino acids 2–185 mapping within the A/B domain, G-20 (rabbit polyclonal) raised against residues 281–300 within the D domain, F-10 (mouse monoclonal) and HC-20 (rabbit polyclonal) raised against residues 576–595 within the F domain—were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). AER-308 (mouse monoclonal) raised against the D/E boundary (residues 283–339) was from Abcam (Cambridge, UK).

Anti-CaM mouse monoclonal antibody was purchased from Upstate (Lake Placid, NY). MAB1501R mouse monoclonal anti-actin was from Chemicon (Temecula, CA). Peroxidase-labeled secondary antibody was from Pierce (Rockford, IL) and anti-rabbit IgG agarose from Sigma.

2.3. Peptides

2.3.1. ERα17p

A first batch of ERα17p peptide (sequence: P_{295}LMIKRSKKNSLA-LSLT_{311}) was gratefully produced by UCB-Bioproducts (Brussels, Belgium) for preliminary studies. The second preparation was synthesized in the Natural Substances laboratory of Meurice Institute (Brussels, Belgium) by the Atherton and Sheppard solid phase peptide synthesis method (Atherton and Sheppard, 1989) on an Advanced ChemTech 90 apparatus. Briefly, 0.25 mM of Fmoc-Thr(But)-Wang resin (0.5 mM/g) was used as solid support. Fmoc-Leu-OPfp, Fmoc-Ser(OBut)-Dhbt, Fmoc-Leu-OPfp, Fmoc-Ala-OPfp, Fmoc-Leu-OPfp, Fmoc-Ser(OBut)-Dhbt, Fmoc-Asn(Trt)-OH, Fmoc-Lys(Boc)-OPfp, Fmoc-Lys(Boc)-OPfp, Fmoc-Ser(OBut)-Dhbt, Fmoc-Arg(Pmc)-OH, Fmoc-Lys(Boc)-OPfp, Fmoc-Ile-OH, Fmoc-Met-Dhbt, Fmoc-Leu-OPfp and Fmoc-Pro-OPfp (0.75 mM) were successively coupled in anhydrous DMF (45 min for Dhbt and OPfp, under N_2) after Fmoc cleavage (20% piperidine in DMF). Couplings were carried out in the presence of HOBt (0.75 mM) for OPfp esters. Activation method with HBTU (0.75 mM, 10 min) (Knorr et al., 1989) was used for Fmoc amino acids under free form in the presence of DIPEA (1.5 mM). All couplings were controlled step by step by analytical Kaiser test (Kaiser et al., 1970). Final cleavage of Wang resin and protecting groups was achieved with a solution of TFA in the presence of scavengers (TFA/H_2O/ethanedithiol/TIS; 94/2.5/2.5/1.0 v/v, 4 h, under N_2). ERα17p was then extracted after resin filtration and lyophilized.

2.3.2. ERα17p analogs

ERα17pAA (sequence: P_{295}LMIKRS$\mathbf{A_{302}A_{303}}$NSLALSLT_{311}) and ERα17pGG (sequence: P_{295}LMIKRS$\mathbf{G_{302}G_{303}}$NSLALSLT_{311}) were synthesized according to the same procedure using Fmoc-Ala-OPfp or Fmoc-Gly-OPfp, respectively.

Molecular weights and sequences of peptides were gratefully confirmed by the University of Ghent, Belgium (J. Van Beeumen and B. Devreese) by MALDI-TOF and/or MALDI-TOF-TOF mass spectrometry. Purity was checked by analytical RP-HPLC achieved on a Waters HPLC system (Waters 600 Pump and Controller, Waters 2487 Dual λ Detector, Waters 746 Data Module) using a C_8 RP-HPLC column (4.6 mm × 300 mm, 5 μm particle size, 300 Å pore size). Mobile phases consisted of appropriate mixtures of B (60% acetonitrile:40% H_2O:0.1% TFA, v/v) and A (H_2O:0.1% TFA, v/v).

2.3.3. CaM kinase II peptide

This peptide, used as a positive control for CaM binding and CaM-dependent kinase II activity measurement (sequence: L_{290}KKFNARRKLK-GAILTTMLA_{309}), was obtained from Calbiochem/Novabiochem (Nottingham, UK).

2.4. Plasmids

2.4.1. pcDNA3-ERwt

HEO ERα cDNA (1.8 kp fragment *Eco*RI from vector pSG5-HEO) provided by P. Chambon (Illkirch, France) (Green et al., 1986) was cloned downstream of the CMV promoter into the *Eco*RI site of pcDNA3 vector (Invitrogen; Carlsbad, CA).

2.4.2. pcDNA3-ERΔERα17p

This construction was obtained from pcDNA3-ERwt by deleting the region coding for the amino acid sequence P_{295}-T_{311}. PCR, used for that purpose (Imai et al., 1991), was performed using Expand Long Template PCR System (Roche Applied Science) with C-terminal 5′-gcttggccaaaggttggcagc-3′ and N-terminal 5′-gccgaccagatggtcagtgcc-3′ primers. PCR product was purified using Wizard SV Gel and PCR Clean-Up System (Promega) and self-ligated (Imai et al., 1991) after blunt-ending with Klenow fragment.

2.4.3. Vit-tk-Luc

Original plasmids tkLuc(puc18-) containing the tk promoter (thymidine kinase) and pVit-tk-Luc containing the regulatory region from −331 nt to −86 nt of Xenopus vitellogenin A2 gene (Vit) were both provided by M. Pons (Montpellier, France). The plasmid used in the current study was constructed by cloning the *Bam*HI-*Hin*dIII 174 bp fragment containing the tk promoter into the *Bgl*II-*Hin*dIII sites of pGL3-Basic (containing the firefly luciferase gene; Promega). Subsequently, the *Bgl*II-*Hin*dIII 476 bp containing the Vit regulatory region was cloned in BglII-HindIII sites upstream the luciferase gene. Construction was verified by sequencing.

2.4.4. pRL-tk

Plasmid containing the *Renilla* luciferase gene under the control of the tk promoter was purchased from Promega.

2.5. Cell culture

MCF-7, T47D, IBEP-1, IBEP-3, BT-20, MDA-MB-231, MDA-MB-453, Evsa-T, HS-578T, SKBR-3 and MVLN cell lines (from our cancer cell line bank) were maintained in a cell incubator at 37 °C in humid atmosphere at 5% CO_2, and propagated in Earle's based minimal essential medium (EMEM) supplemented with Phenol Red, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated FBS (all reagents from Invitrogen/Gibco). Experiments were performed in EMEM without Phenol Red and containing 10% charcoal-stripped FBS in order to avoid any potential estrogenic interference. The change of medium was performed 48 h before treatment, except for growth measurement and transient transfections (24 h).

2.6. CaM-Sepharose binding assays

CaM-Sepharose 4B was equilibrated beforehand in 10 mM Tris–HCl pH 7.5 containing 100 mM KCl and 1 μM $CaCl_2$.

2.6.1. ER binding

hER (∼2.5 pmol in 1 ml of equilibration buffer containing 1 mg/ml BSA) was labeled with 1 nM [^{3}H]E_2 alone (total binding), or in the presence of a 200 fold excess of E_2 (non-specific binding). Labeled [^{3}H]E_2–ER complex was incubated overnight with 50 μl of CaM-Sepharose suspension (50% slurry), in presence or absence of anti-ER antibodies (100 ng/ml), CaM antagonists (10 μM) or investigated peptides (10 μM). CaM-Sepharose was then washed three times with 1 μM Ca^{2+} (fractions 1–3) and eluted with 10 mM EDTA (fractions 4–6). ER concentration was determined by measuring radioactivity ([^{3}H]E_2 labeled samples) using a Wallac 1409 liquid scintillation counter (Perkin Elmer; Boston, MA) or by Western blotting using F-10 anti-ER antibody (see below).

2.6.2. Peptide binding

ERα17p and its analogs, dissolved in the equilibration buffer (10 μM; 100 μl) were incubated with CaM-Sepharose or with Sepharose 4B (control for assessment of potential non-specific binding) (50 μl, 50% slurry; 2 h, 4 °C). After removing unbound peptides (supernatant) matrixes were washed two times with 1 μM Ca^{2+} and bound peptides were eluted with 10 mM EDTA. Twenty μl of unbound and bound peptide were then separated on a 16.5% Tris–Tricin gel and revealed by Sypro Ruby staining (Bio-Rad; Hercules, CA).

2.7. Dansylated CaM fluorescence assay

Direct interaction of ERα17p with CaM was assessed using dansylated CaM (dansyl-CaM) in a fluorescence assay (Kincaid et al., 1982). The CaM kinase II

peptide was used as positive control. Samples were prepared in quartz cuvettes in a final volume of 3 ml of 10 mM Tris–HCl, pH 7.5, 0.1 mM $CaCl_2$, 150 mM NaCl. Fluorescence measurements were recorded on a Perkin Elmer LS-5B luminescence spectrometer at room temperature with both emission and excitation slits at 5 nm. The excitation wavelength was 340 nm and the mixture was scanned over an emission wavelength range of 400–600 nm. Specificity of the fluorescence signal was demonstrated by its abrogation in the presence of 500 μM EGTA.

2.8. Assay of CaM-dependent cAMP phosphodiesterase activity

The assay of cAMP phosphodiesterase activity was performed with [8-^{3}H]cAMP (Amersham Biosciences) as substrate, under optimal conditions i.e. 100 μM [8-^{3}H]cAMP (0.1 μCi), 100 ng (3.6 U) CaM, 2 mU of beef heart phosphodiesterase (EC. 3.1.4.17) in 40 mM Tris–HCl pH 7.4, 4 mM DTT, 5 mM $MgCl_2$. Basal activity (CaM independent) was determined by adding 1 mM EGTA to the medium. Tritiated AMP formed during the incubation was converted into tritiated adenosine by 5′-nucleotidase from snake venom. Nucleoside products were separated from unreacted substrate by batch elution with Dowex anion exchange resin and tritiated adenosine level determined by liquid scintillation counting (Rowlands et al., 1990; McCague et al., 1994).

2.9. MTT cytotoxicity assay

MCF-7 cells were seeded in 96-well plates (3000 cells/well). After treatment (6, 16, 24 or 48 h) with CaM antagonists (calmidazolium chloride, W-7 or ophiobolin A) or ERα17p, cell viability was assayed by exposure to 0.03% MTT dissolved in EMEM without Phenol Red (90 min, 37 °C). After medium removal, produced formazan was dissolved in DMSO (1 h, room temperature, under agitation) for measurement by spectrometry at 550 nm using a Microplate Autoreader EL309 (BIO-TEK Instruments; Winooski, VT).

2.10. Co-immunoprecipitation

MCF-7 cells were incubated for 2 h with CaM antagonists or with ERα17p in serum free condition. They were then washed with PBS (40 mM Na_2HPO_4, 10 mM KH_2PO_4, 120 mM NaCl, pH 7.2) and lysed in TBS (50 mM Tris–HCl, 150 mM NaCl, pH 7.5) containing 0.5% NP-40, 0.2 mM Ca^{2+}, 0.6 mM PMSF and 0.3 mM TPCK. Lysates were clarified by centrifugation and immunoprecipitated (Laios et al., 2005). Briefly, for each sample, cell lysates corresponding to 250 μg of total protein were pre-cleared using agarose-coupled anti-rabbit IgG (45 μl, 50% slurry, 2 h, 4 °C) and were thereafter incubated with IgG anti-ER antibody (HC-20; 2 μg, overnight, 4 °C). ER-antibody complexes were precipitated by agarose-coupled anti-rabbit IgG (45 μl, 50% slurry, 2 h, 4 °C). After washing, pellets, were suspended in 60 μl electrophoresis buffer (LDS Sample 1× buffer; Invitrogen) and boiled for 5 min. ER and associated CaM levels were measured by Western blot analysis (see below).

2.11. ER measurement (whole cell [^{3}H]E_2 binding assay)

In order to determine ER binding capacity, MCF-7 cells were plated in 24-well dishes. Two days later, cells were exposed for 24 h to increasing amounts of ERα17p (1–50 μM). Medium was then removed and exposed to 1 nM of [^{3}H]E_2 in serum-free EMEM without Phenol Red. Additional wells were filled with a 500-fold excess of unlabelled E_2 for non-specific binding (NSB) measurement. After 45 min of incubation, the medium was again removed and the monolayer washed three times with PBS. Cell-bound radioactivity was finally extracted with ethanol (30 min, room temperature) and measured by scintillation counting.

For binding kinetics analysis, a similar protocol was used, except that MCF-7 cells were incubated for different periods of time (6–48 h, see Section 3) in presence of ERα17p or ERα17p analogs at 10 μM, without or with 1 μM MG-132. Thereafter, cells were exposed to [^{3}H]E_2 at several concentrations (from 0.05 to 1 nM) without or with excess of unlabelled E_2; two additional wells were used for assessing protein concentration in cell extracts. Binding kinetics parameters (i.e. dissociation constant K_d and binding capacity B_{max}) were determined by Scatchard plot analysis (Scatchard, 1949).

2.12. Western blot analysis

After treatment with CaM antagonists, E_2, ERα17p or ERα17p analogs, MCF-7 cells were washed with PBS before lysis in TBS containing 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM NaF, 0.1 mM orthovanadate, 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 (15 μg protein per lane) to electrophoresis on 4–12% SDS-PAGE gel (Invitrogen). Separated proteins were electrotransferred onto Hybond ECL nitrocellulose membrane (Amersham) using a semi-dry blotting apparatus (Bio-Rad). Non-specific sites were blocked by pretreatment with 5% non-fat dry milk in TBS containing 0.05% Tween 20 (2 h, room temperature). Membranes were then incubated overnight at 4 °C with anti-ER (D-12 or F-10), anti-CaM or anti-actin (MAB1501R) antibodies (dilutions of 1:1000, 1:3000, 1:1000 and 1:7500, respectively). Detection was performed by chemiluminescence, using a peroxidase-coupled secondary antibody (dilution of 1:2000, 1.5 h, room temperature) and Western Pico Detection system (Pierce). Immunoblots were visualized using FLA-3000 camera (Fuji; Tokyo, Japan). Densitometric analyses were performed using Aida software.

2.13. ER immunofluorescence staining

MCF-7 cells were plated on sterile round glass coverslips in 12-well dishes. Two days after seeding, cells were incubated with ERα17p at 10 μM. After 24 h exposure, cell monolayers were rinsed with Dulbecco's PBS (DPBS) and fixed at 4 °C with phosphate-buffered 4% paraformaldehyde (PAF). Following fixation, PAF was changed for DPBS. Demonstration of ER by immunofluorescence was achieved as described previously (Brohee et al., 2000). In short, cell monolayers were rinsed several times with PBS containing 0.1% Triton X-100. Before exposure to the primary antibody, cells were preincubated for 20 min in PBS containing 0.05% casein (PBS-cas; Sigma) and 50 mM NH_4Cl to prevent non-specific adsorption of immunoglobulins. Cells were then exposed for 1 h to HC-20 antibody diluted 1:50 in PBS-cas. Thereafter, they were incubated for 30 min in the presence of a dextran polymer conjugated with both peroxidase and antibodies raised against rabbit immunoglobulins (EnVision™, DAKO Diagnostics, Heverlee, Belgium). The next step consisted in a 30 min incubation with rabbit antiserum raised against horseradish peroxidase (Laboratory of Hormonology, Marloie, Belgium), followed by a 30 min incubation in presence of biotinylated swine anti-rabbit immunoglobulins antibodies (from DAKO). Texas Red labeling was completed by exposing cells for 30 min to Texas Red-conjugated streptavidin (Vector Laboratories, Burlingame, CA). After thorough rinses in PBS, the coverslips were mounted on glass slides using commercial anti-fading medium (Vectashield®; Vector Laboratories). The cell preparations were examined on a Leitz Orthoplan microscope equipped with a Ploem system for epi-illumination. Texas Red fluorescence was examined at an excitation wavelength of 596 nm and an emission wavelength of 615 nm.

2.14. ERE-dependent transcriptional activity

2.14.1. MVLN (MCF-7 stably transfected with pVit-tk-Luc reporter plasmid) (Pons et al., 1990)

After treatment with CaM antagonists, E_2, ERα17p or ERα17p analogs, cells were washed 2 times with PBS. Luciferase activity was measured in cell lysates by luminometry using Luciferase Assay System (Promega) according to a protocol described previously (Seo et al., 2000).

2.14.2. Evsa-T (ER-negative, PgR-positive breast cancer cell line) (Borras et al., 1997)

Cells, seeded in 6-well plates in the absence of antibiotics, were transiently transfected with ER constructions (pcDNA3-ERwt or pcDNA3-ERΔERα17p) and reporter plasmids Vit-tk-Luc (ERE-mediated expression of firefly luciferase) and pRL-tk (basal expression of *Renilla* luciferase). Two μg/well of pcDNA3-ERwt or pcDNA3-ERΔERα17p and Vit-tk-Luc, and 400 ng/well of pRL-tk were transfected using FuGENE 6 (Roche Applied Science). Sixteen hours after transfection, cells were treated for 24 additional hours with 0.1 nM E_2 and/or 10 μM ERα17p. Cells were then lysed and luciferase activities measured using

Dual-Luciferase Reporter Assay System (Promega). Luciferase responses were normalized with respect to *Renilla* luciferase activity.

2.15. Cells growth measurement

ER-positive (MCF-7, T47D, IBEP-1, IBEP-3, BT-20) (de Longueville et al., 2005) and ER-negative (MDA-MB-231, MDA-MB-453, Evsa-T, HS-578T, SKBR-3) breast cancer cell lines (for review: Lacroix and Leclercq, 2004) were seeded in 96-well plates (3000 cells/well). Cells were then treated with E_2 at 0.1 nM or ERα17p and ERα17p analogs at 10 μM for 24, 48, and 72 h. Cell growth was measured by crystal violet staining (Journe et al., 2004). Briefly, cell cultures were gently washed once with PBS, fixed with 1% glutaraldehyde in PBS (15 min, room temperature) and stained with 0.1% crystal violet (w/v in ddH_2O; 30 min, room temperature). After removal of excess dye by rinsing under gently running tap water, cell-bound crystal violet was extracted with 1% Triton X-100 (v/v in ddH_2O; room temperature, under agitation) and measured by spectrometry at 550 nm.

2.16. Statistical analysis

Statistical analysis was performed by ANOVA, followed by post-hoc Tukey test to assess differences between selected groups (SPSS software). Significance level was arbitrarily set at a *p* value of 0.05.

3. Results

3.1. Ability of ERα17p to antagonize ER-CaM association

When this study was initiated, the precise location of the CaM binding site in ER was still not established. Yet, an amino acid sequence located at the boundary between D and E domains of ER ($P_{293}SPLMIKRSKKNSLALS_{309}$) was suspected to be involved in receptor interaction with CaM (Castoria et al., 1988; Bouhoute and Leclercq, 1995). Thus, we utilized a panel of antibodies raised against different ER epitopes (Fig. 1A) in order to evaluate the implication of this part of the receptor in its association with CaM. By doing this, we found that only antibodies raised against the junction between D and E domains (i.e. G-20 and AER-308) impeded the binding of a highly purified preparation of recombinant hER to CaM-Sepharose (Fig. 1B). Of note, no effect of these two antibodies on $[^3H]E_2$ binding parameters was recorded by Scatchard plot analysis (data not shown), excluding the possibility that they might interfere with receptor binding properties. These observations prompted us to synthesize a peptide containing the suspected CaM binding motif (ERα17p; $P_{295}LMIKRSKKNSLALSLT_{311}$; Fig. 1C). It should be stressed here that our option has been recently validated by other investigators who indeed demonstrated the implication of this motif in CaM recruitment, by using various ER mutant constructions (Li et al., 2005).

As could be expected, ERα17p inhibited ER-CaM association, since at 10 μM it decreased the binding of hER to CaM-Sepharose (Fig. 2). In this respect, its effect was similar to that of two CaM inhibitors (calmidazolium and ophiobolin A) taken as reference compounds. Surprisingly and for unknown reasons, another CaM inhibitor (W-7) failed to exhibit antagonistic activity (Fig. 2). Of note, these observations were reproduced with $[^3H]E_2$-bound hER (inhibition of ER binding to immobilized CaM: 54, 12, 61 and 41% for calmidazolium, W-7, ophiobolin A and ERα17p, respectively, all compounds at 10 μM). Hence, under our experimental conditions, E_2 did not modify the ability of ERα17p to inhibit the binding of ER to CaM-Sepharose.

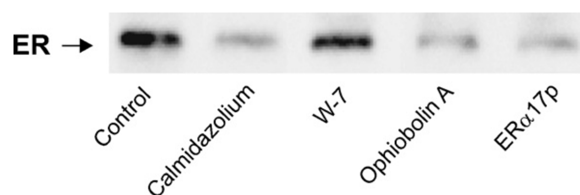


Fig. 2. Inhibition of ER–CaM-Sepharose binding by ERα17p. Preparations of recombinant hER were incubated with CaM-Sepharose in the absence (control) or presence of 10 μM of CaM antagonists (calmidazolium, W-7 and ophiobolin A) or of ERα17p. After washing, matrixes were eluted with EDTA and levels of released ER assessed by Western blot analysis using F-10 anti-ER antibody. The figure is representative of three independent experiments.

The ability of ERα17p to directly interact with CaM was checked by a fluorescence-based binding assay using dansylated CaM. In this assay, a CaM kinase II peptide, used as a reference, produced a characteristic increase in fluorescence intensity (Fig. 3A). ERα17p also induced an increase of dansylated CaM fluorescence. However, as compared to the CaM kinase II peptide, a four-fold higher concentration of ERα17p (1 μM *versus* 0.25 μM) was required to achieve a noticeable augmentation of the fluorescence signal, indicating a lower binding affinity of ERα17p *versus* the reference peptide. Accordingly, we observed

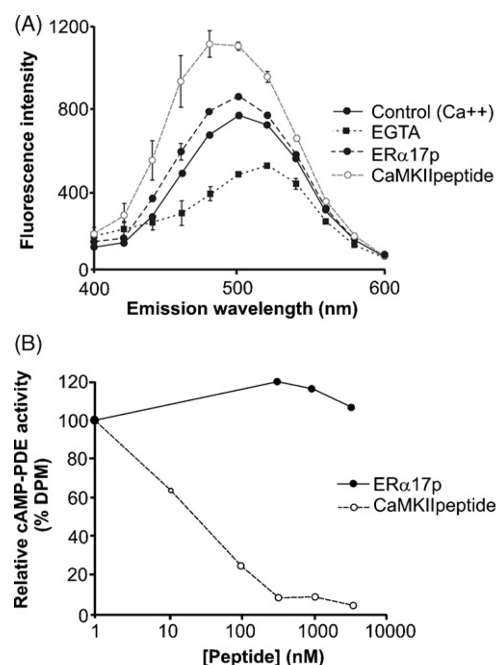


Fig. 3. Lack of strong interaction between ERα17p and CaM. (A) Measurement of ERα17p–CaM binding by a dansylated CaM fluorescence assay. Fluorescence intensity was measured in the absence (control; ●) or presence of EGTA (■). ERα17p at 1 μM (●) and CaM kinase II peptide (positive control; ○) at 0.25 μM produced a significant increase in fluorescence intensity. (B) Cyclic AMP phosphodiesterase activity measurement. Increasing amounts of ERα17p (●) and CaM kinase II peptide (○) were added to a phosphodiesterase preparation in the presence of CaM and $[^3H]cAMP$ (substrate). After incubation, tritiated adenosine produced by $[^3H]cAMP$ hydrolysis and subsequent cleavage of $[^3H]AMP$ was measured by liquid scintillation counting.

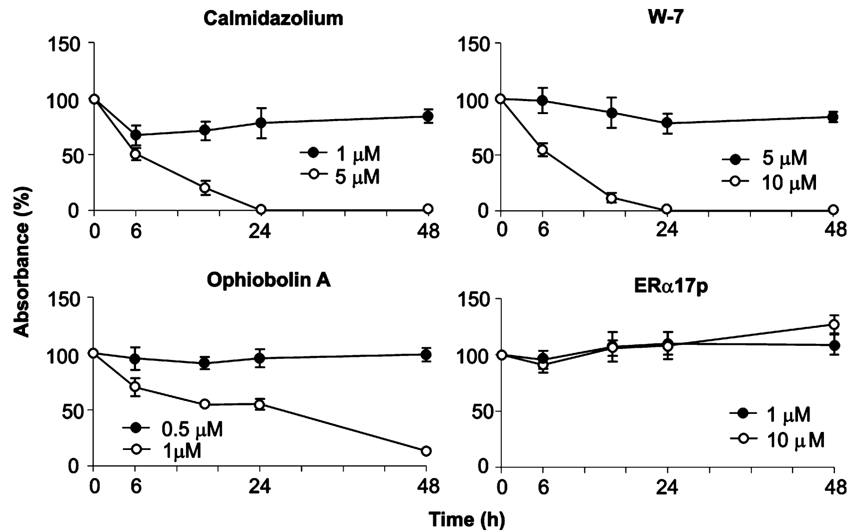


Fig. 4. Effect of CaM inhibitors and ERα17p on MCF-7 cell viability. MCF-7 cells were incubated for 6, 16, 24 and 48 h in the absence (control = 100%) or presence of calmidazolium (1 and 5 μM), W-7 (5 and 10 μM), ophiobolin A (0.5 and 1 μM) or ERα17p (1 and 10 μM). After treatment, cell viability was measured by MTT assay. Results were expressed in % absorbance (mean ± S.D.) of control. Measurements were performed in sixplicate. The figure refers to an experiment performed twice.

that ERα17p up to 20 μM had no antagonistic activity toward a CaM-dependent cyclic AMP phosphodiesterase, while the CaM kinase II peptide acted as a potent inhibitor at largely lower concentrations (IC50 of 22 ± 5 nM; $n = 3$; Fig. 3B). Altogether, our observations suggested that ERα17p antagonizes ER-CaM interaction without drastically affecting CaM-mediated processes. Toxicity tests confirmed this interpretation. Thus, MTT assays performed on MCF-7 cells exposed to ERα17p at 10 μM failed to disclose a decrease of metabolic activity even after prolonged treatment, while classical CaM inhibitors (calmidazolium, W-7 and ophiobolin A) displayed a detectable inhibitory effect at equivalent or lower concentrations (Fig. 4). Of note, ERα17p at 10 μM induced after 48 h an increase of mitochondrial activity which, as shown below, could be relevant to a growth stimulation.

Interference of ERα17p with the formation of intracellular ER-CaM complexes was finally established by co-immunoprecipitation with an anti-ER antibody in MCF-7 cells extracts (Fig. 5). After two hours of cell treatment with

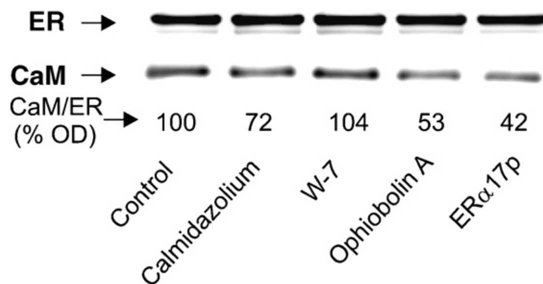


Fig. 5. Inhibition of endogenous ER-CaM association by ERα17p. MCF-7 cells were treated for 2 h with CaM antagonists (1 μM calmidazolium, 5 μM W-7 and 0.5 μM ophiobolin A) or with 10 μM of ERα17p. After cell lysis, ER-CaM complexes were co-immunoprecipitated with F-10 anti-ER antibody. Level of ER and CaM were then revealed by Western blot. The figure shows an immunoblot representative of two independent experiments and densitometric analysis values (mean ratio of CaM/ER optical densities). Control, untreated cells.

ERα17p, CaM level decreased in the co-immunoprecipitate while the amount of ER remained constant, demonstrating thereby the dissociation of ER-CaM complexes. The observation of a similar phenomenon with cells treated with calmidazolium and ophiobolin A at non-toxic concentrations confirmed the validity of this assay. Of note, these treatments affected neither ER level nor that of CaM in whole cell lysates (data not shown).

3.2. ERα17p-induced ER down regulation and ERE-dependent transcription

Exposure of MCF-7 cells to ERα17p for 24 h resulted in a dose-dependent loss of their capacity to accumulate $[^3H]E_2$ (IC50 = 5.3 μM; $n = 3$; Fig. 6A). As shown by Scatchard plot analysis (Fig. 6B), this phenomenon was associated with a decrease of binding sites (B_{max}) without substantial change in the dissociation constant of the binding reaction (K_d). A similar observation, suggestive of a down regulation process, was also recorded with ophiobolin A at 0.5 μM (data not shown). We were therefore not surprised to observe that ERα17p-induced loss of estrogen binding capacity was correlated with a decrease of receptor level, which was evidenced by both Western blot analysis (Fig. 6D) and immunofluorescence microscopy (Fig. 6E). However, the loss of binding capacity persisted even when ER degradation was prevented by the proteasome inhibitor MG-132 (Fig. 6C), as shown previously for ER ligands (Laios et al., 2005; Seo et al., 2006). In fact, binding capacity decreased largely before ER degradation (i.e. after one hour of treatment; data not shown), suggesting that it may result from a conformational change preceding the proteasomal degradation. Hence, early ER conformational changes commonly induced by ligands seem also to be provoked by ERα17p.

A link between agonist-induced proteasomal degradation of ER and its ERE-dependent transcriptional activity has

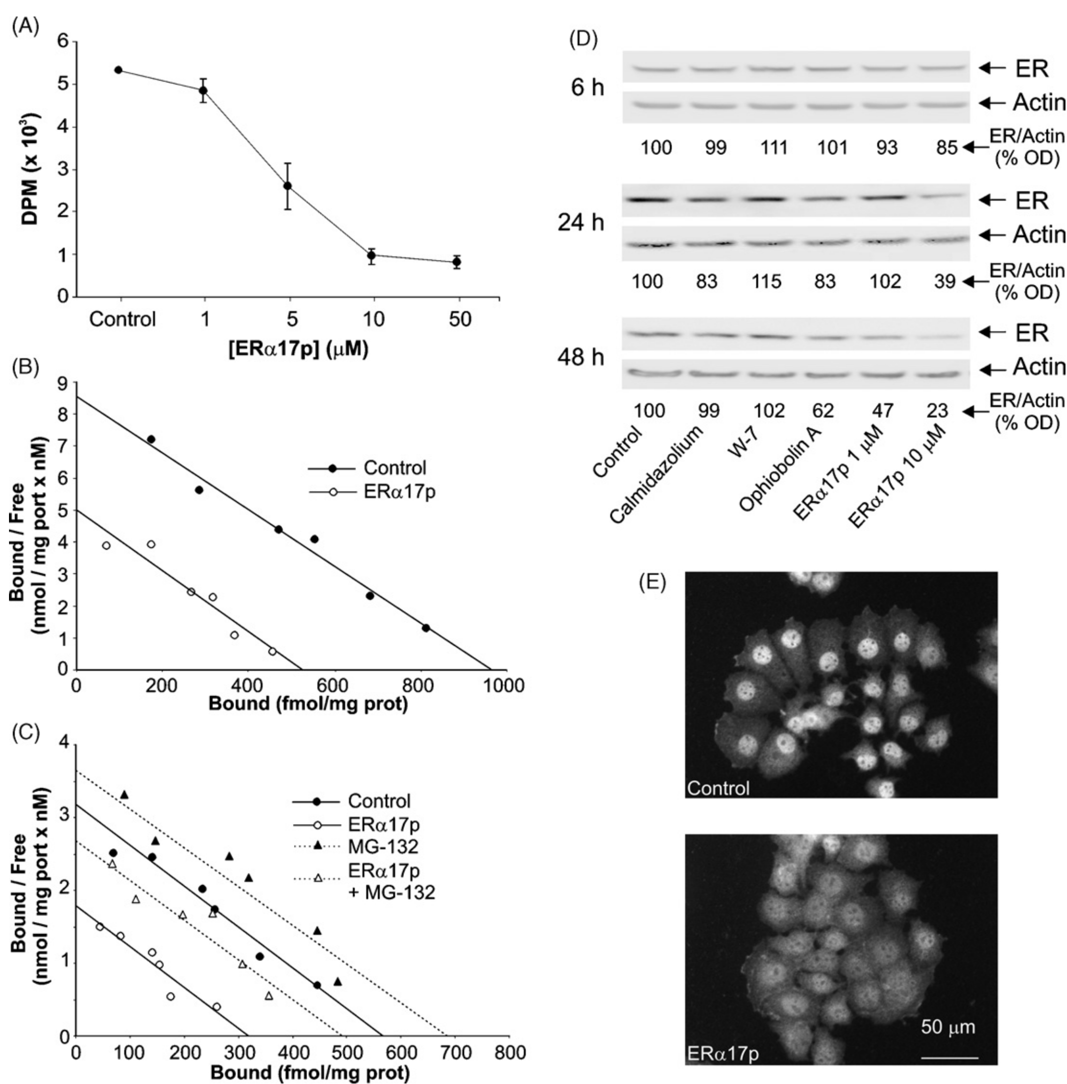


Fig. 6. ERα17p-induced ER down regulation. (A) Loss of estrogen binding capacity. MCF-7 cells were incubated with ERα17p at concentrations ranging from 1 to 50 μM. After 24 h of treatment, binding capacity was measured by incubation of the cells with [^{3}H]E_2 (whole cell assay). Each point represents the mean ± S.D. of three separate experiments with determinations performed in duplicate. (B) Influence on [^{3}H]E_2 binding parameters. MCF-7 cells were incubated for 24 h in the absence (control) or presence of 10 μM ERα17p before exposure to [^{3}H]E_2. K_d and B_{max} parameters were determined by Scatchard plot analysis. Representative results of an experiment performed four times, where K_d ranged from 0.08 to 0.26 nM (B_{max} decrease induced by ERα17p was 52 ± 12% as compared to control). (C) Loss of estrogen binding capacity in the presence of MG-132. MCF-7 cells were incubated for 6 h in the absence (control) or presence of 10 μM ERα17p with or without MG-132 at 1 μM before exposure to [^{3}H]E_2. Binding parameters were determined as described above. The figure is representative of four independent experiments in which K_d ranged from 0.09 to 0.23 nM. In the absence of proteasome inhibitor, the B_{max} decrease induced by ERα17p was 41 ± 7% as compared to control. In the presence of MG-132, B_{max} was decreased by 43 ± 16%. (D) Western blot analysis. MCF-7 cells were cultured for 6, 24 and 48 h in the absence (control) or presence of either a CaM antagonist (1 μM calmidazolium, 5 μM W-7 and 0.5 μM ophiobolin A) or ERα17p at 1 and 10 μM. Western blots were carried out using D-12 anti-ER and MAB1501R anti-actin antibodies. Immunoblots are representative of three independent experiments. Densitometric analysis values (ratio of ER/actin ODs) are given and expressed as percentages of control. (E) ER demonstration by immunofluorescence microscopy. Cells were incubated for 24 h in the absence (control) or presence of 10 μM ERα17p. ER was demonstrated by immunofluorescence staining using HC-20 antibody.

been proposed in the recent literature (Reid et al., 2003; Metivier et al., 2003; Yan et al., 2003; Laios et al., 2005). In the current study, experiments performed with MCF-7 cells stably transfected with a Vit-tk-Luc reporter gene (MVLN cells) exposed to 10 μM of ERα17p revealed that the ERα17p-induced ER down regulation was associated with a stimulation of ERE-dependent transactivation (mean = 179 ± 11%; n = 8; Fig. 7). This result was in sharp contrast with the finding that, under the same experimental conditions, CaM inhibitors either were ineffective or produced a slight inhibition of ER-mediated gene transactivation. Hence, ERα17p seemed to be endowed with an estrogen-like activity without exerting a major effect on CaM. This was confirmed by the fact that ERα17p at 10 μM enhanced the expression of two estrogen-dependent endogenous genes after 24 h of treatment (progesterone receptor and Ps2 mRNA; 226 and 272%, respectively). Of note, co-treatment with E_2 and ERα17p failed to produce any additive effect. On the other hand, calmodulin inhibitors, probably because of their cytotoxicity, slightly decreased E_2-enhanced transcription.

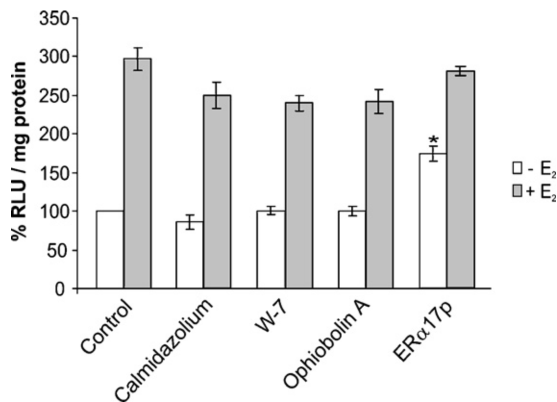


Fig. 7. Influence of ERα17p on ERE-dependent transcription. MVLN cells were incubated for 24 h in the absence (control) or presence of CaM antagonist (1 μM calmidazolium, 5 μM W-7 and 0.5 μM ophiobolin A) or ERα17p at 10 μM, with or without 0.1 nM E_2. Luciferase activity was assayed in cellular extracts by luminometry and emitted light signals were expressed in arbitrary units (relative luciferase units, RLU) per mg protein. Data refer to the mean value (±S.D.) of, at least, three independent experiments with measurements performed in duplicate and are given as percentage of controls. (*) Significantly higher as compared to control.

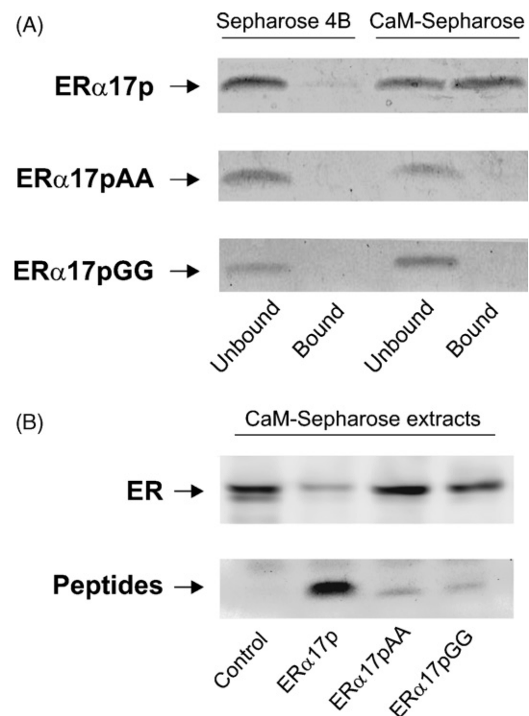


Fig. 8. Failure of ERα17p analogs to inhibit ER-CaM association. (A) ERα17p, ERα17pAA and ERα17pGG all at 10 μM were incubated with Sepharose 4B (for assessment of non-specific adsorption) or CaM-Sepharose matrixes. After washing, and elution with EDTA, bound and unbound peptides were separated by electrophoresis and revealed by Sypro Ruby staining. The figure, representative of two independent experiments, demonstrates that, in contrast to ERα17p, the two analogs do not bind to CaM-Sepharose. Note also, for all peptide, the absence of adsorption on Sepharose. (B) Lack of antagonism of ERα17p analogs toward ER-CaM association. Preparations of recombinant hER were incubated with CaM-Sepharose in the absence (control) or presence of 10 μM of ERα17p, ERα17pAA and ERα17pGG. After washing, matrixes were eluted with EDTA and levels of bound ER assessed by Western blot analysis using F-10 anti-ER antibody (top), while the level of bound peptides was revealed by silver staining (bottom). The figure is representative of two independent experiments.

3.3. Assessment of CaM-independent mechanisms in ERα17p-mediated ER regulation

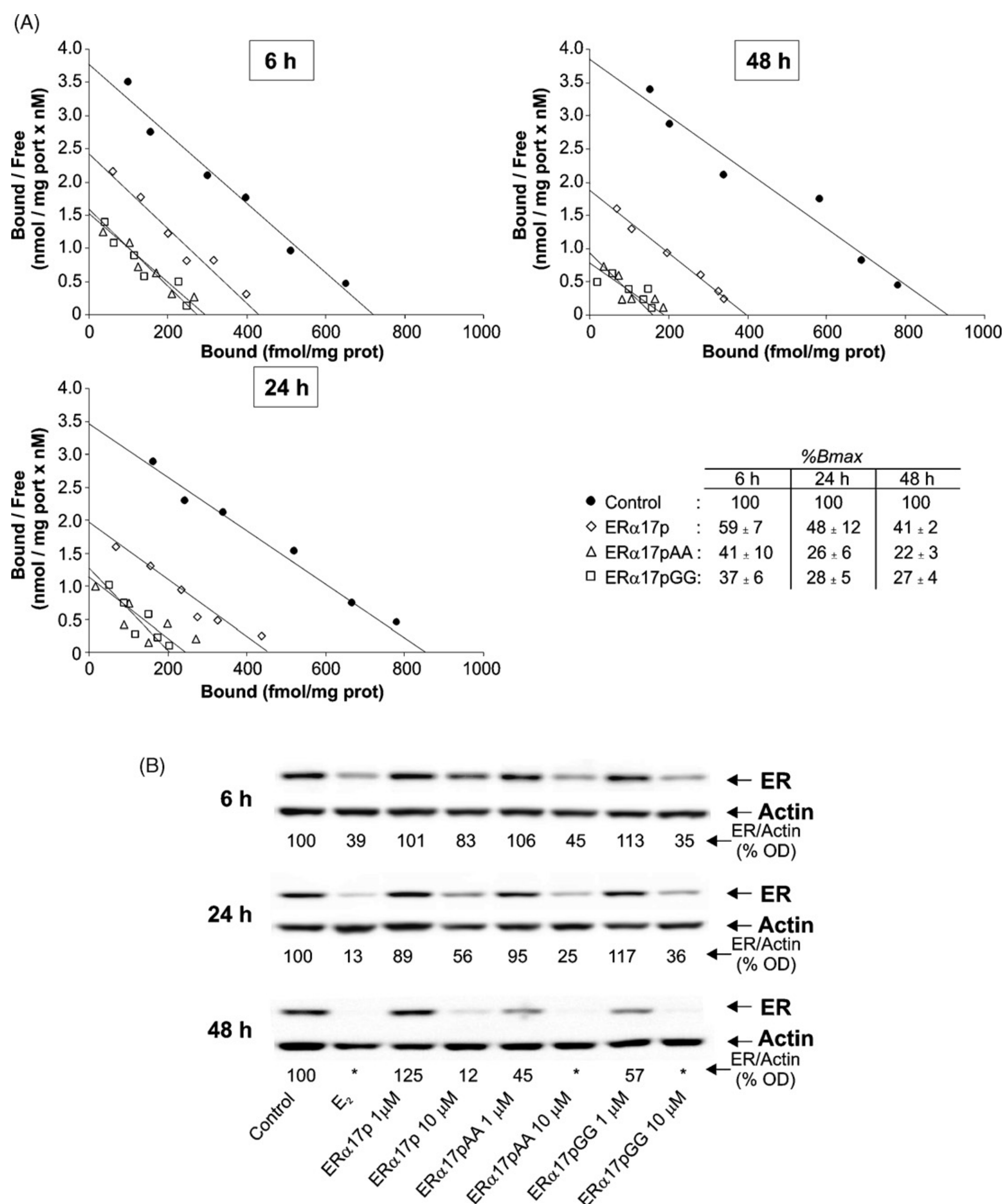
Data described above led us to concentrate on the actual involvement of CaM in the mechanism underlying the agonistic activity of ERα17p. The ER motif corresponding to ERα17p is, indeed, a region undergoing post-translational modifications critical for various physiological processes which are not necessarily CaM-dependent i.e. phosphorylation on S_{305} (Wang et al., 2002) and T_{311} (Lee and Bai, 2002), acetylation on K_{299}, K_{302} and K_{303} (Wang et al., 2001), SUMOylation (Sentis et al., 2005), proteolysis ($K_{299}RSKK_{303}$ motif) (Seielstad et al., 1995). Furthermore, the ERα17p sequence harbors a nuclear localization signal (NLS; $K_{299}RSKK_{303}$) (Picard et al., 1990; Ylikomi et al., 1992).

In order to assess the importance of CaM in ERα17p-mediated agonistic responses, we synthesized two analogs where lysines K_{302} and K_{303}, reported to be essential for CaM binding to ER (Garcia Pedrero et al., 2002), were substituted by alanines (ERα17pAA) or glycines (ERα17pGG). As expected, these two analogs failed to bind CaM (Fig. 8A and B bottom) and, unlike ERα17p, did not compete with ER for the binding to CaM-Sepharose (Fig. 8B top). We considered, therefore, that they were appropriate tools for unraveling potential CaM-independent interactions involved in ERα17p-induced ER activation.

As inferred from the measurement of [^{3}H]E_2 binding capacity (Fig. 9A) and ER level (Fig. 9B) in MCF-7 cells, ERα17pAA and ERα17pGG induced ER down regulation with a slightly higher efficiency than ERα17p. These analogs also increased ERE-dependent transactivation in MVLN cells (Fig. 10), providing evidence that the effect of ERα17p on ER regulation was not strictly related to a dissociation of ER-CaM complexes. According to this view, one may assume that ERα17p did not strongly affect the initial CaM-dependent steps of ER-mediated transactivation but rather, the subsequent events culminating in ER degradation. ERα17pAA and ERα17pGG would exclusively promote the latter events.

3.4. Growth stimulation of ER-positive cell lines induced by ERα17p and its analogs

At this stage, it appeared crucial to evaluate the possibility that ERα17p and its analogs might exert an effect on the growth of ER-positive cell lines. As illustrated in Fig. 11, the three peptides at 10 μM stimulated the proliferation of a panel of five ER-positive breast cancer cell lines (MCF-7, T47D, IBEP-1, IBEP-3 and BT-20) (E_2 was included as a positive control). Stimulatory properties of these peptides varied among investigated cell lines, reflecting their biological differences (Lacroix and Leclercq, 2004); peptide analogs appeared to be more efficient than ERα17p in this regard. By contrast, the peptides did not affect the proliferative activity of ER-negative cell lines (MDA-MB-231, MDA-MB-453, Evsa-T, HS-578T and



%Bmax	6 h	24 h	48 h
Control	100	100	100
ERα17p	59 ± 7	48 ± 12	41 ± 2
ERα17pAA	41 ± 10	26 ± 6	22 ± 3
ERα17pGG	37 ± 6	28 ± 5	27 ± 4

Fig. 9. Down regulation of ER by ERα17p analogs. MCF-7 cells were cultured during 6, 24 and 48 h in the absence (control) or presence of ERα17p, ERα17pAA or ERα17pGG all at 10 μM. (A) $[^3H]E_2$ binding parameters (K_d, B_{max}) were determined by whole cell binding assay and Scatchard plot analysis. The figure is representative of three experiments where K_d ranged from 0.08 to 0.33 nM. B_{max} values (mean ± S.D.) are given as percentage of control. (B) ER level determination. Cells were incubated with 0.1 nM of E_2 or either 1 and 10 μM of ERα17p, ERα17pAA or ERα17pGG. ER and actin levels were then assayed by Western blot analysis using F-10 and MAB1501R antibodies. Immunoblots are representative of three independent experiments. Densitometric analysis values (ratio of ER/actin ODs) are given and expressed as percentages of control. (*) OD not quantified.

SKBR-3). These experiments unambiguously established the implication of ER in the mechanism of action of ERα17p and excluded any possibility that the observations reported above might result from non-specific effects. The finding that a panel of eight peptides derived from DNA and ligand binding domains of ER (i.e. R_{211}-M_{220}, P_{222}-K_{231}, D_{332}-L_{346}, K_{363}-V_{377}, K_{417}-A_{431}, S_{456}-H_{474}, A_{491}-L_{504} and D_{538}-A_{551}) failed to similarly stimulate MCF-7 cell growth (data not shown) is another proof of the specificity.

3.5. Molecular mechanism underlying ERα17p-induced activation of ER

We finally addressed the mechanism involved in the action of ERα17p on ER-positive cell lines. For that purpose, a transient transfection of the ER-negative, PgR-positive breast cancer cell line Evsa-T (Borras et al., 1997) with an ER variant lacking the ERα17p motif (ERΔERα17p) was performed. This transfection resulted in an enhanced expression of a co-transfected

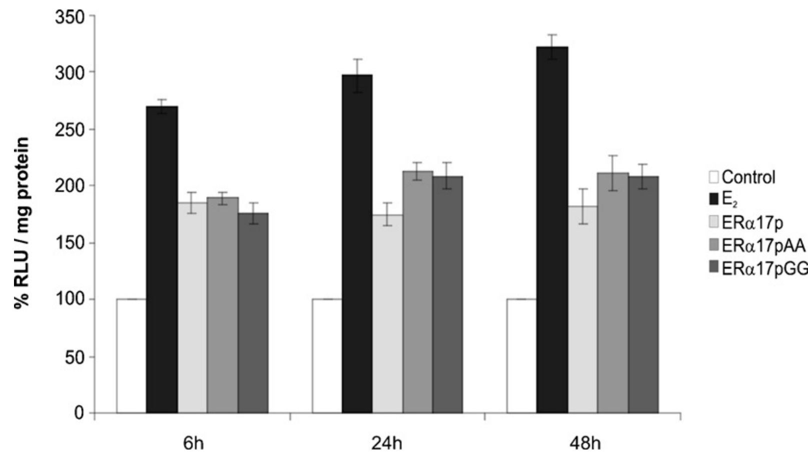


Fig. 10. ERE-dependent transcription induced by ERα17p analogs. MVLN cells were incubated for 6, 24 and 48 h in the absence (control) or presence of 0.1 nM of E_2 or ERα17p, ERα17pAA and ERα17pGG all at 10 μM. Luciferase activity was assayed in cellular extracts by luminometry and emitted light signals were expressed in arbitrary units (relative luciferase units, RLU) per mg protein. Data refer to the mean value ± S.D. of three independent experiments with measurements performed in duplicate and are given as percentage of controls. All compounds induced a significant increase as compared to the corresponding controls.

ERE-dependent reporter gene (Fig. 12). Strikingly, reporter gene expression in cells co-transfected with this ER variant was amplified neither by E_2, nor by ERα17p, while control cells transfected with wild type ER displayed an usual behavior (as described in MVLN cells). Hence, agonistic responses described here seem to be due to the disruption of a repressive effect to which the P_{295}-T_{311} motif largely contributes. This statement provides an explanation to data reported by Li et al. (2005), which showed that various deletions in the D/E border region were associated with high basal, E_2-insensitive transcriptional activity.

4. Discussion

Small synthetic peptides harboring the canonical motif of co-activators (*LxxLL*) or co-repressors (*Lxx*(*H*/*I*)*Ixx*(*L*/*I*)) have been found to enhance or repress, respectively, ERE-dependent transcription (Xu et al., 2002; Iannone et al., 2004; Rodriguez et al., 2004; Shao et al., 2004). Hence, peptides that may interfere with co-regulator recruitment have proven to be useful tools for evaluating the mechanism by which ER mediates transcription. Data reported here extend this concept to peptides exhibiting ER regulatory motifs. Indeed, we clearly show that ERα17p

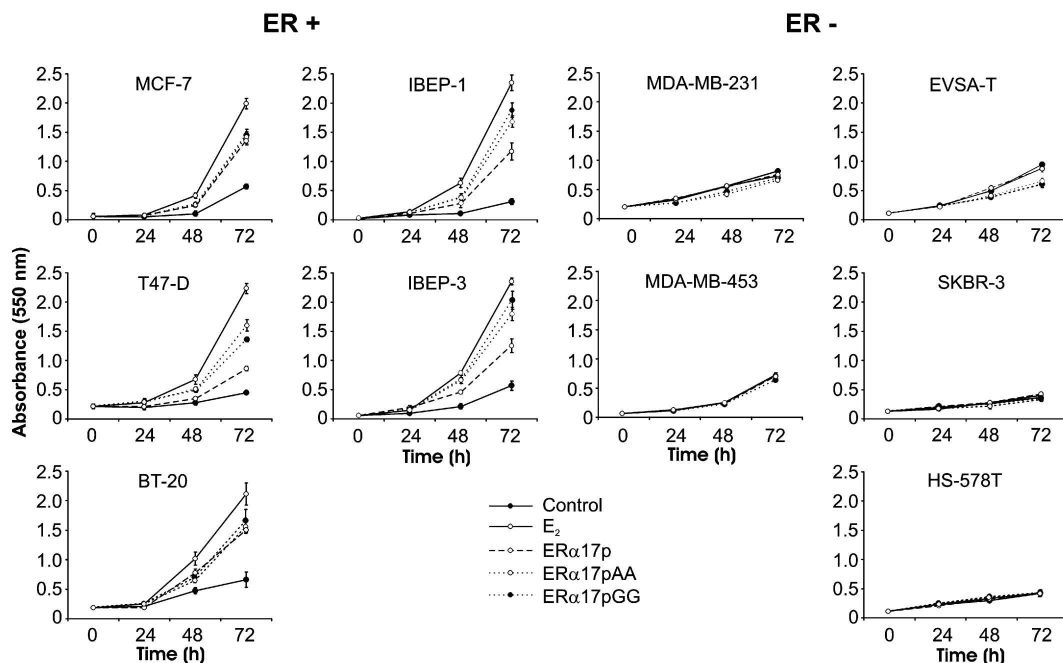


Fig. 11. Influence of ERα17p and ERα17p analogs on growth of breast cancer cell lines. ER-positive and ER-negative breast cancer cell lines were grown for 24, 48 and 72 h in the absence (control) and presence of 0.1 nM of E_2 or of ERα17p, ERα17pAA and ERα17pGG all at 10 μM. Cell growth was measured by crystal violet staining. Measurements were performed in sixplicate. Data refer to the mean value ± S.D. of a representative experiment performed three times.

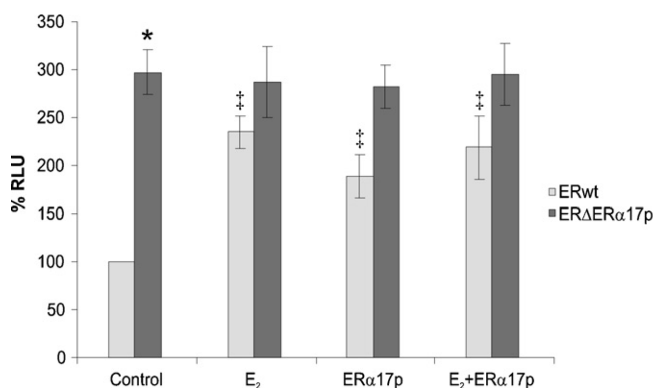


Fig. 12. Constitutive activity of an ER mutant deleted for ERα17p motif. Evsa-T cells were transiently transfected with ERwt or ERΔERα17p in the presence of Vit-tk-Luc (ERE-mediated expression of firefly luciferase) and pRL-tk (basal expression of *Renilla* luciferase) reporter plasmids. Cells were then incubated for 24 h in the absence (control) or presence of 0.1 nM of E_2 or/and 10 μM ERα17p. Luciferase activities were assayed in cellular extracts by luminometry and emitted light signals were normalized with respect to *Renilla* luciferase activity. Values are expressed as percentage of that obtained with ERwt-transfected cells (control). Data refer to mean values (±S.D.) of at least four independent experiments. (* and ‡) Significantly higher as compared to untreated ERwt-transfected cells. No significant difference was noted between RLU values measured in ERΔERα17p-transfected cells.

increases ER-mediated transcription in breast cancer cell lines, leading to a stimulation of their growth.

The P_{295}-T_{311} sequence of ER, to which ERα17p corresponds, is a multifaceted regulatory platform subjected to several post-translational modifications, indicative of its importance in ER regulation. By a computer-based protein modeling approach (Jacquot et al., 2007), we recently identified a putative regulatory intramolecular interaction between a motif included in the P_{295}-T_{311} sequence (aa 301-311) and the helix H4 which is located within a region known to recruit co-regulators (H3-H5) (Warnmark et al., 2002; Kong et al., 2005). Hence, the estrogen-like activity of ERa17p might stem from its ability to interfere with this regulatory mechanism. According to our data, CaM by specifically interfering with the P_{295}-T_{311}–H4 interaction would most probably abrogate an associated specific repressive effect, perhaps with concomitant release of bound co-repressor(s) and/or recruitment of co-activator(s). This view is supported by the fact that other peptides outside the region covered by ERα17p do not have similar impact on cell growth. Ongoing studies are carried out to assess this hypothesis.

CaM has been reported to be overexpressed in breast cancers, suggesting its implication in the development of the disease (Singer et al., 1976). This concept is somehow supported by our investigations, inasmuch as a high amount of CaM may favor the activation of ER and lead *in fine* to an increased ER-mediated transactivation and an enhanced cell proliferation. This postulate may also explain why CaM antagonists impede the growth of breast cancer cells and synergistically augment the antiproliferative action of antiestrogens (Strobl et al., 1994; Wei et al., 1983).

Conventional CaM inhibitors maintain CaM in an inactive status and most probably abrogate its binding to ER, blocking thereby the association of the latter with ERE, i.e. the initial step of the transcription cycle. In contrast to CaM inhibitors which have been reported to impede ER-mediated transactivation (Biswas et al., 1998; Garcia Pedrero et al., 2002; Li et al., 2005), synthetic peptides containing the P_{295}-T_{311} sequence would confer to ER an activated conformation similar to that induced by CaM, even if they are unable to directly interact with CaM.

K_{302} and K_{303} of ER have been reported to be crucial for the recruitment of CaM (Garcia Pedrero et al., 2002). Our finding that the ERα17pAA and ERα17pGG analogs of ERα17p fail to associate with CaM confirms the importance of these two lysines. The basic ε-amino group in these amino acids may be taken as responsible for their capacity to attract CaM. In this view, a missense point mutation causing the substitution of an arginine (R) for K_{303} has been detected in hyperplastic and neoplastic breast lesions (Fuqua et al., 2000; Herynk and Fuqua, 2004; Conway et al., 2005). Studies performed with MCF-7 cells transfected with such a K303R mutant revealed that this mutation induces an increase of sensitivity to estradiol with regard to cell proliferation. This mutation also favors the recruitment of the TIF-2 co-activator at lower E_2 concentration than the wild type receptor (Fuqua et al., 2000). Since arginine is more basic than lysine, one may surmise that the hyperactivity of the K303R mutant may be relevant to a higher ability to attract CaM, with as a consequence a facilitated conversion of the receptor into its activated form.

ER-mediated transcription involves cyclic association/dissociation of the receptor with/from specific targets (i.e. EREs, heat shock proteins, histone acetyltransferases of the CBP/p300 family, transcription factors, ubiquitin ligases. . .) (for review: Leclercq et al., 2006; Nawaz and O'Malley, 2004). The ability of CaM to enhance ER binding to EREs suggests its involvement in the association of the receptor with the promoter regions of target genes. One may logically postulate that this initial step of the transactivation cycle requires a receptor where all regulatory motifs (zinc fingers, AF-1 and AF-2 domains. . .) are fully operational. The ability of CaM to prevent ER ubiquitination (Li et al., 2006) should logically meet this requirement. This protective effect of CaM would progressively vanish, leading to the transfer of the receptor to the ubiquitin/proteasome system. Hence, CaM-induced activation would only affect the stability of the receptor at a step beyond its anchorage to the promoter. In the context of this model, synthetic peptides like ERα17p would favor this activation process. Whether proteasomal degradation of ER provokes the emergence of small peptides with regulatory properties similar to those of ERα17p is another issue that we are currently investigating. Indeed, the production of such peptides may be a key determinant of the basal (ligand-independent) transcriptional activity of ER.

As illustrated here, an ER deletion mutant lacking the P_{295}-T_{311} sequence exhibits an enhanced estrogen independent transcriptional activity. Interestingly, this property has already been recorded for other mutations affecting this region of the receptor (Li et al., 2005). The potential lack of operative NLS in such mutants appears, therefore, not detrimental to ER-mediated transcription, in agreement with studies previously reported

(Picard et al., 1990; Ylikomi et al., 1992). ER activation most probably favors the exposure of other NLS or similar structural motifs promoting the transport of the receptor across the nuclear membrane.

The design of peptide mimics able to modify the ability of ER to recruit co-regulators has been proposed as an alternative to conventional estrogen- or antiestrogen-based hormonotherapy (Rodriguez et al., 2004; Shao et al., 2004). This approach, which would be especially valuable for the treatment of pathologies associated with receptor insensitivity to ligand binding, is still in its infancy. Our study supports such a pharmacological approach, even if the concentration of ERα17p and its analogs required for the onset of a significant response is high. This drawback, also found for *LxxLL* mimics (Iannone et al., 2004) most probably results from a low cellular uptake. Thus, there is an obvious need for an improvement of pharmacokinetic properties resulting in a better intracellular penetration of small peptides with potential therapeutic activity. Synthesis of non-peptide hydrophobic drugs with similar competitive properties for ER (Rodriguez et al., 2004; Shao et al., 2004) is another approach to avoid this pitfall.

The current study is mainly focused on ER transcriptional activity in breast cancer cells. One may wonder whether similar results would be obtained with other estrogen target cells, in particular cells where distribution and shuttling of ER are different. Beside, recent investigations have clearly shown that ER does not only elicit genomic responses, but also triggers rapid, so-called non-genomic responses *via* signal transduction cascades (Cheskis, 2004; Singh and Kumar, 2005; Evinger and Levin, 2005; Kampa and Castanas, 2006). Studies aimed at knowing whether ERα17p also activates ER-mediated non-genomic responses are planned in the near future. Furthermore, future investigations should also examine the potential impact of intracellular Ca^{2+} spikes on the mechanisms described here (co-treatment with Ca^{2+} channel modulators). Hence, our observations open new avenues in the study of the mechanisms by which estrogens (and most probably other steroid hormones) provoke specific responses in target tissues.

Acknowledgments

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References

Atherton, E., Sheppard, R.C., 1989. Solid Phase Peptide Synthesis: A Practical Approach. IRL Press, Oxford.

Biswas, D.K., Reddy, P.V., Pickard, M., Makkad, B., Pettit, N., Pardee, A.B., 1998. Calmodulin is essential for estrogen receptor interaction with its motif and activation of responsive promoter. J. Biol. Chem. 273, 33817–33824.

Borras, M., Lacroix, M., Legros, N., Leclercq, G., 1997. Estrogen receptor-negative/progesterone receptor-positive Evsa-T mammary tumor cells: a model for assessing the biological property of this peculiar phenotype of breast cancers. Cancer Lett. 120, 23–30.

Bouhoute, A., Leclercq, G., 1995. Modulation of estradiol and DNA binding to estrogen receptor upon association with calmodulin. Biochem. Biophys. Res. Commun. 208, 748–755.

Brohee, R., Nonclercq, D., Journe, D.N., Toubeau, G., Falmagne, P., Leclercq, G., Heuson-Stiennon, J.A., Laurent, G., 2000. Demonstration of estrogen receptors and of estrogen responsiveness in the HKT-1097 cell line derived from diethylstilbestrol-induced kidney tumors. In Vitro Cell. Dev. Biol. Anim. 36, 640–649.

Castoria, G., Migliaccio, A., Nola, E., Auricchio, F., 1988. In vitro interaction of estradiol receptor with Ca2+-calmodulin. Mol. Endocrinol. 2, 167–174.

Cheskis, B.J., 2004. Regulation of cell signalling cascades by steroid hormones. J. Cell. Biochem. 93, 20–27.

Conway, K., Parrish, E., Edmiston, S.N., Tolbert, D., Tse, C.K., Geradts, J., Livasy, C.A., Singh, H., Newman, B., Millikan, R.C., 2005. The estrogen receptor-alpha A908G (K303R) mutation occurs at a low frequency in invasive breast tumors: results from a population-based study. Breast Cancer Res. 7, R871–R880.

de Longueville, F., Lacroix, M., Barbuto, A.M., Bertholet, V., Gallo, D., Larsimont, D., Marcq, L., Zammatteo, N., Boffe, S., Leclercq, G., Remacle, J., 2005. Molecular characterization of breast cancer cell lines by a low-density microarray. Int. J. Oncol. 27, 881–892.

Evans, R.M., 1988. The steroid and thyroid hormone receptor superfamily. Science 240, 889–895.

Evinger III, A.J., Levin, E.R., 2005. Requirements for estrogen receptor alpha membrane localization and function. Steroids 70, 361–363.

Fuqua, S.A., Wiltschke, C., Zhang, Q.X., Borg, A., Castles, C.G., Friedrichs, W.E., Hopp, T., Hilsenbeck, S., Mohsin, S., O'Connell, P., Allred, D.C., 2000. A hypersensitive estrogen receptor-alpha mutation in premalignant breast lesions. Cancer Res. 60, 4026–4029.

Garcia Pedrero, J.M., Del Rio, B., Martinez-Campa, C., Muramatsu, M., Lazo, P.S., Ramos, S., 2002. Calmodulin is a selective modulator of estrogen receptors. Mol. Endocrinol. 16, 947–960.

Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J.M., Argos, P., Chambon, P., 1986. Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature 320, 134–139.

Herynk, M.H., Fuqua, S.A., 2004. Estrogen receptor mutations in human disease. Endocr. Rev. 25, 869–898.

Iannone, M.A., Simmons, C.A., Kadwell, S.H., Svoboda, D.L., Vanderwall, D.E., Deng, S.J., Consler, T.G., Shearin, J., Gray, J.G., Pearce, K.H., 2004. Correlation between in vitro peptide binding profiles and cellular activities for estrogen receptor-modulating compounds. Mol. Endocrinol. 18, 1064–1081.

Imai, Y., Matsushima, Y., Sugimura, T., Terada, M., 1991. A simple and rapid method for generating a deletion by PCR. Nucleic Acids Res. 19, 2785.

Jacquot, Y., Gallo, D., Leclercq, G., 2007. Estrogen receptor alpha—Identification by a modeling approach of a potential polyproline II recognizing domain within the AF2 region of the receptor that would play a role of prime importance in its mechanism of action. J. Steroid Biochem. Mol. Biol. 104, 1–10.

Journe, F., Chaboteaux, C., Dumon, J.C., Leclercq, G., Laurent, G., Body, J.J., 2004. Steroid-free medium discloses oestrogenic effects of the bisphosphonate clodronate on breast cancer cells. Br. J. Cancer 91 (9), 1703–1710.

Kaiser, E., Colescott, R.L., Bossinger, C.D., Cook, P.I., 1970. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. Anal. Biochem. 34, 595–598.

Kampa, M., Castanas, E., 2006. Membrane steroid receptor signaling in normal and neoplastic cells. Mol. Cell. Endocrinol. 246, 76–82.

Kincaid, R.L., Vaughan, M., Osborne Jr., J.C., Tkachuk, V.A., 1982. Ca2+-dependent interaction of 5-dimethylaminonaphthalene-1-sulfonyl-calmodulin with cyclic nucleotide phosphodiesterase, calcineurin, and troponin I. J. Biol. Chem. 257, 10638–10643.

Knorr, R., Trzeciak, A., Bannwarth, W., Gillessen, D., 1989. New coupling reagents in peptide chemistry. Tetrahedron Lett. 30, 1927–1930.

Kong, E.H., Heldring, N., Gustafsson, J.A., Treuter, E., Hubbard, R.E., Pike, A.C., 2005. Delineation of a unique protein–protein interaction site on the surface of the estrogen receptor. Proc. Natl. Acad. Sci. U.S.A. 102, 3593–3598.

Lacroix, M., Leclercq, G., 2004. Relevance of breast cancer cell lines as models for breast tumours: an update. Breast Cancer Res. Treat. 83, 249–289.

Laios, I., Journe, F., Nonclercq, D., Vidal, D.S., Toillon, R.A., Laurent, G., Leclercq, G., 2005. Role of the proteasome in the regulation of estrogen receptor alpha turnover and function in MCF-7 breast carcinoma cells. J. Steroid. Biochem. Mol. Biol. 94, 347–359.

Leclercq, G., Lacroix, M., Laios, I., Laurent, G., 2006. Estrogen receptor alpha: impact of ligands on intracellular shuttling and turnover rate in breast cancer cells. Curr. Cancer Drug Targets 6, 39–64.

Lee, H., Bai, W., 2002. Regulation of estrogen receptor nuclear export by ligand-induced and p38-mediated receptor phosphorylation. Mol. Cell. Biol. 22, 5835–5845.

Li, L., Li, Z., Sacks, D.B., 2003. Calmodulin regulates the transcriptional activity of estrogen receptors. Selective inhibition of calmodulin function in subcellular compartments. J. Biol. Chem. 278, 1195–1200.

Li, L., Li, Z., Sacks, D.B., 2005. The transcriptional activity of estrogen receptor-alpha is dependent on Ca2+/calmodulin. J. Biol. Chem. 280, 13097–13104.

Li, L., Li, Z., Howley, P.M., Sacks, D.B., 2006. E6AP and calmodulin reciprocally regulate estrogen receptor stability. J. Biol. Chem. 281, 1978–1985.

Li, Z., Joyal, J.L., Sacks, D.B., 2001. Calmodulin enhances the stability of the estrogen receptor. J. Biol. Chem. 276, 17354–17360.

McCague, R., Rowlands, M.G., Grimshaw, R., Jarman, M., 1994. Evidence that tamoxifen binds to calmodulin in a conformation different to that when binding to estrogen receptors, through structure-activity study on ring-fused analogues. Biochem. Pharmacol. 48, 1355–1361.

Metivier, R., Penot, G., Hubner, M.R., Reid, G., Brand, H., Kos, M., Gannon, F., 2003. Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. Cell 115, 751–763.

Nawaz, Z., O'Malley, B.W., 2004. Urban renewal in the nucleus: is protein turnover by proteasomes absolutely required for nuclear receptor-regulated transcription? Mol. Endocrinol. 18, 493–499.

Picard, D., Kumar, V., Chambon, P., Yamamoto, K.R., 1990. Signal transduction by steroid hormones: nuclear localization is differentially regulated in estrogen and glucocorticoid receptors. Cell Regul. 1, 291–299.

Pons, M., Gagne, D., Nicolas, J.C., Mehtali, M., 1990. A new cellular model of response to estrogens: a bioluminescent test to characterize (anti) estrogen molecules. Biotechniques 9, 450–459.

Reid, G., Hubner, M.R., Metivier, R., Brand, H., Denger, S., Manu, D., Beaudouin, J., Ellenberg, J., Gannon, F., 2003. Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. Mol. Cell. 11, 695–707.

Rodriguez, A.L., Tamrazi, A., Collins, M.L., Katzenellenbogen, J.A., 2004. Design, synthesis, and in vitro biological evaluation of small molecule inhibitors of estrogen receptor alpha coactivator binding. J. Med. Chem. 47, 600–611.

Rossini, G.P., 1994. The quaternary structures of untransformed steroid hormone receptors: an open issue. J. Theor. Biol. 166, 339–353.

Rowlands, M.G., Parr, I.B., McCague, R., Jarman, M., Goddard, P.M., 1990. Variation of the inhibition of calmodulin dependent cyclic AMP phosphodiesterase amongst analogues of tamoxifen; correlations with cytotoxicity. Biochem. Pharmacol. 40, 283–289.

Scatchard, G., 1949. The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51, 660–672.

Seielstad, D.A., Carlson, K.E., Kushner, P.J., Greene, G.L., Katzenellenbogen, J.A., 1995. Analysis of the structural core of the human estrogen receptor ligand binding domain by selective proteolysis/mass spectrometric analysis. Biochemistry 34, 12605–12615.

Sentis, S., Le Romancer, M., Bianchin, C., Rostan, M.C., Corbo, L., 2005. SUMOylation of the estrogen receptor alpha hinge region regulates its transcriptional activity. Mol. Endocrinol. 19, 2671–2684.

Seo, H.S., Larsimont, D., Ma, Y., Laios, I., Leclercq, G., 2000. Regulation of estrogen receptor levels by ligand-induced release of compound(s) in MCF-7 cells. Mol. Cell. Endocrinol. 164, 19–29.

Seo, H.S., DeNardo, D.G., Jacquot, Y., Laios, I., Vidal, D.S., Zambrana, C.R., Leclercq, G., Brown, P.H., 2006. Stimulatory effect of genistein and apigenin on the growth of breast cancer cells correlates with their ability to activate ER alpha. Breast Cancer Res. Treat. 99, 121–134.

Shang, Y., Hu, X., DiRenzo, J., Lazar, M.A., Brown, M., 2000. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell 103, 843–852.

Shao, D., Berrodin, T.J., Manas, E., Hauze, D., Powers, R., Bapat, A., Gonder, D., Winneker, R.C., Frail, D.E., 2004. Identification of novel estrogen receptor alpha antagonists. J. Steroid. Biochem. Mol. Biol. 88, 351–360.

Singer, A.L., Sherwin, R.P., Dunn, A.S., Appleman, M.M., 1976. Cyclic nucleotide phosphodiesterases in neoplastic and nonneoplastic human mammary tissues. Cancer Res. 36, 60–66.

Singh, R.R., Kumar, R., 2005. Steroid hormone receptor signaling in tumorigenesis. J. Cell. Biochem. 96, 490–505.

Strobl, J.S., Peterson, V.A., Woodfork, K.A., 1994. A survey of human breast cancer sensitivity to growth inhibition by calmodulin antagonists in tissue culture. Biochem. Pharmacol. 47, 2157–2161.

Wang, C., Fu, M., Angeletti, R.H., Siconolfi-Baez, L., Reutens, A.T., Albanese, C., Lisanti, M.P., Katzenellenbogen, B.S., Kato, S., Hopp, T., Fuqua, S.A., Lopez, G.N., Kushner, P.J., Pestell, R.G., 2001. Direct acetylation of the estrogen receptor alpha hinge region by p300 regulates transactivation and hormone sensitivity. J. Biol. Chem. 276, 18375–18383.

Wang, R.A., Mazumdar, A., Vadlamudi, R.K., Kumar, R., 2002. P21-activated kinase-1 phosphorylates and transactivates estrogen receptor-alpha and promotes hyperplasia in mammary epithelium. Embo J. 21, 5437–5447.

Warnmark, A., Treuter, E., Gustafsson, J.A., Hubbard, R.E., Brzozowski, A.M., Pike, A.C., 2002. Interaction of transcriptional intermediary factor 2 nuclear receptor box peptides with the coactivator binding site of estrogen receptor alpha. J. Biol. Chem. 277, 21862–21868.

Wei, J.W., Hickie, R.A., Klaassen, D.J., 1983. Inhibition of human breast cancer colony formation by anticalmodulin agents: trifluoperazine, W-7, and W-13. Cancer Chemother. Pharmacol. 11, 86–90.

Xu, H.E., Stanley, T.B., Montana, V.G., Lambert, M.H., Shearer, B.G., Cobb, J.E., McKee, D.D., Galardi, C.M., Plunket, K.D., Nolte, R.T., Parks, D.J., Moore, J.T., Kliewer, S.A., Willson, T.M., Stimmel, J.B., 2002. Structural basis for antagonist-mediated recruitment of nuclear co-repressors by PPARalpha. Nature 415, 813–817.

Yan, F., Gao, X., Lonard, D.M., Nawaz, Z., 2003. Specific ubiquitin-conjugating enzymes promote degradation of specific nuclear receptor coactivators. Mol. Endocrinol. 17, 1315–1331.

Ylikomi, T., Bocquel, M.T., Berry, M., Gronemeyer, H., Chambon, P., 1992. Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. Embo J. 11, 3681–3694.