The Transcriptional Activity of Estrogen Receptor-α Is Dependent on Ca^{2+}/Calmodulin*

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Estrogen receptors function as ligand-activated transcription factors that regulate the expression of target genes to affect processes as diverse as reproduction, development, and general metabolism (1, 2). Estrogen receptor-α (ERα) shares structural organization with other members of the nuclear receptor family. It contains an N-terminal region that harbors a ligand-independent transcriptional activation function (AF-1); a core DNA binding domain, containing two highly conserved zinc finger motifs that target the receptor to specific DNA sequences known as estrogen response elements (ERE); a hinge region that permits protein flexibility to allow for simultaneous receptor dimerization and DNA binding; and a large C-terminal region that encompasses the ligand binding domain, dimerization interface, and a ligand-dependent activation function (AF-2) (1). Upon ligand binding, ERα undergoes a conformational change that coordinately dissociates corepressors and facilitates recruitment of coactivator proteins to enable transcriptional activation (3). Protein-protein interaction screens have revealed a large group of proteins classified as coactivators on the basis of their ability to enhance ERα action when overexpressed in target cells (3). Some of these proteins have an important role in ERα action and provide functional and physical links between the receptor and the transcriptional apparatus. The precise roles of most of these proteins remain to be determined.

Recent studies have shown that regulation of transcription by nuclear ERα is more complicated than the classical paradigm would predict (reviewed in Refs. 4–6). For example, in some cells both AF-1 and AF-2 are required for maximal transcriptional activities, whereas in others only one is required (7, 8). Calmodulin, a ubiquitous Ca^{2+} sensor protein, appears to participate in ERα transcriptional activity. For example, the cell-permeable calmodulin antagonists CGS9343B and trifluoperazine prevented 17β-estradiol (E2) from stimulating ERα transcription (9, 10). Similarly, we documented that E2 failed to enhance transcriptional activity by ERα in cells transiently transfected with a peptide that specifically inhibits calmodulin function in the nucleus (9). Moreover, interaction with calmodulin seems to be required for ERα to bind the ERE (11, 12) and activate an ERα-responsive promoter (12). However, the strategies employed in these prior studies were nonspecific or indirect, and recent major reviews continue to ignore the possible role of calmodulin in ERα function (1, 13, 14). Therefore, in this work we employed a multifaceted approach to unequivocally document that a direct interaction of ERα with calmodulin is necessary for E2 to stimulate ERα transcriptional activation. We developed a point mutant ERα construct (termed ERαΔCaM) that is unable to bind calmodulin. Despite binding E2 with an affinity 3.5-fold greater than that of wild-type ERα, transcriptional activity of ERαΔCaM was not augmented by E2. Congruent with our prior documentation that ERα binds directly to calmodulin in a Ca^{2+}-dependent manner (15), chelation of intracellular free Ca^{2+} ([Ca^{2+}]i) abrogated E2-stimulated transcriptional activation of ERα. Importantly, a point mutant calmodulin that is unable to bind Ca^{2+} functioned as a dominant negative construct, reducing both basal and E2-stimulated transcriptional activity of ERα. Taken together, these data indicate that calmodulin has an essential role in ERα transcriptional activity.

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1 The abbreviations used are: ERα, estrogen receptor-α; Luc, luciferase; E2, 17β-estradiol; PVDF, polyvinylidene difluoride; [Ca^{2+}]i, intracellular free Ca^{2+}; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl)ester; ERE, estrogen response element; RT-PCR, reverse transcriptase PCR; hnRNA, heterogeneous nuclear RNA; CaM, calmodulin.
**EXPERIMENTAL PROCEDURES**

**Materials**—Tissue culture reagents were purchased from Invitrogen and fetal bovine serum (FBS) was obtained from Biowhittaker. Charcoal-treated FBS was from Cocalico Biologicals Inc. MCF-7 breast epithelial cells and COS-7 green monkey kidney cells were obtained from American Type Culture Collection. Recombinant human osteocalcin (Gla) was purchased from R&D Systems. Transfections were performed using FuGENE 6 according to the manufacturer’s instructions. DNA sequencing was performed with the ABI PRISM 377 DNA Sequencer, using an ABI PRISM 310 DNA sequencer, using an ABI PRISM 310 DNA sequencer.

**ERα Require Calmodulin for Transcription**

**Plasmid Construction**—The truncated ERα cDNAs used in the TnT assays (see Fig. 1C for complete list and specific sequences) were amplified by PCR from pCDNA3-ERα. All 5′-primers contained a BamHI site and all 3′-primers contained an ApaI site. Each PCR fragment was digested with BamHI and ApaI, then inserted into pCDNA3-myc at the same restriction sites.

**Site-directed mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Stratagene). Plasmid pCDNA3-myc-ERα was used as template. The mutant cDNA was amplified with primers 5′-CATGACGGCGCTTGGCACAAG-3′ and the N-terminal primers 5′-AACAGCCTGGCCTTGGC-3′ and 5′-GCCCTTGGTTGACGTCCG-3′, respectively. 7-kilobase fragments were purified from low melting agarose and allowed to self-ligate. The constructs are named ERα289-303, ERα293-310, and ERα298-317, respectively.

**Luciferase Reporter Assay**—For determination of luciferase activity specifically bound to wild-type ERα, activity was quantified by liquid scintillation spectrophotometry. Radiolabeled ERα was prepared as described above. Total RNA was extracted by TRIzol (Invitrogen) and reverse transcribed to cDNA using the iScriptTM cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s instructions. The resulting cDNA was used in subsequent RT-PCR reactions, performed in 1× iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer’s instructions. The relative quantities of RT-PCR products were determined using the iCycler System (Bio-Rad) for 40 cycles (94°C for 10 s, 60°C for 40 s), after an initial 3 min denaturation at 94°C. Serial dilutions of a stock solution of the known template were used to determine the efficiency of the RT-PCR reaction.

**Western Blot Analysis**—Equal amounts of protein lysate were resolved directly by SDS-PAGE and transferred to PVDF membrane. Membrane were blocked with 5% nonfat powdered milk in TBS-T buffer (25 mM Tris, pH 8.0, 140 mM NaCl, 2.5 mM KCl, and 0.05% Tween 20) and probed with anti-ERα, anti-hemagglutinin, or anti-calmodulin antibodies. Complexes were visualized with the appropriate horseradish peroxidase conjugated secondary antibody and developed by enhanced chemiluminescence (ECL).

**In Vitro Transcription and Translation—**[35S]Methionine-labeled ERα were produced with the TnT quick-coupled transcription/translation system according to the manufacturer’s instructions. As described previously, 1 μg of DNA was mixed with 40 μl of TnT-quick master mix and 10 μCi of [35S]methionine (10 μCi/ml, PerkinElmer Life Sciences), then incubated at 30°C for 90 min. Products were confirmed by SDS-PAGE and autoradiography.

**Calmodulin Binding**—In vitro binding assays were performed by incubating 10 μl of reticulocyte lysate containing [35S]methionine-label- ed wild-type ERα and [35S]methylated bovine serum albumin (BSA) ligand (wild-type ERα and the ERα mutants indicated in the figure legend) with calmodulin-Sepharose beads in 1 ml of Buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 0.1% protease inhibitor mixture (Sigma), and 1 mM phenylmethylsulfonyl fluoride) in the presence or absence of 1 mM CaCl2. Sepharose without calmodulin was used as control. After 4 h at 4°C, beads were washed four times with Buffer A containing 1 mM CaCl2 or 1 mM EGTA. Proteins were analyzed by SDS-PAGE, and [35S]methylated-labeled ERα was detected by autoradiography.

**pDNA3 containing the indicated ERα cDNA plasmids, namely ERα-1–595, ERα-1–270, ERα-300–595, ERα-180–595, ERα-180–353, ERα-180–253, ERα-248–317, or ERα-180–317 (numbers in parentheses correspond to the amino acid residues of ERα), were transiently transfected into COS-7 cells. Forty-eight hours after transfection, cells were washed with PBS, lysed with 1% Buffer A containing 1 mM CaCl2, and sonicated, containing 1 μl CaCl2, and analyzed by SDS-PAGE and Western Blotting as described above.

**ERα Binding Assay**—A whole cell ligand binding assay was used to measure the estrogen binding capacity of ERα (20). COS-7 cells, plated in 24-well culture dishes, were transfected with wild-type or mutant ERα. After 24 h, the culture medium was replaced with phenol red-free medium. After another 24 h, followed by 2 h incubation with 10 nM [3H]estradiol (PerkinElmer Life Sciences). A 200-fold excess of diethylstilbestrol was added to one set of cells to distinguish between specific and nonspecific binding. The cells were washed once with phosphate-buffered saline supplemented with 1 mg/ml bovine serum albumin and once with phosphate-buffered saline alone. The cells were lysed in buffer containing 1% Triton X-100, 7.5 mM KCl, 1 mM CaCl2, 5 mM sodium molybdate, 0.4 mM KCl, 1 mM monoglycerol, and 2 mM leupeptin, and disrupted by freeze-thawing three times. The amount of bound radioactivity was quantified by liquid scintillation spectrophotometry. Radioactivity specifically bound to wild-type ERα (150,000 to 300,000 cpm) was 10-fold greater than nonspecific binding. A set of cells processed in parallel was used to verify by Western blotting that cells expressed equivalent levels of ERα.

**Quantitative Real-time RT-PCR—**Quantitative RT-PCR was performed on the iCycler Platform real-time PCR detection system (Bio-Rad) to examine the effect of E2 on the estrogen responsive gene pS2. MCF-7 cells transfected with pCDNA3 or pCDNA1234, and COS-7 cells transfected with wild-type ERα or ERαCaM was incubated with or without E2 as described above. Total RNA was extracted by TRIzol (Invitrogen) according to the manufacturer’s protocol. After quantification in a spectrophotometer, 2 μg of RNA was reverse-transcribed to cDNA in a total volume of 40 μl using the iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s instructions. The resulting cDNA was used in subsequent RT-PCR reactions, performed in 1× iQ SYBR Green Supermix (Bio-Rad) with 5 pmol forward and reverse primers (21). The PCR conditions were as follows: for forward primer, 5′-TCTGCTGAGATTCAACATTAAAGGAGAAG-3′ (start position 3697, within the intron); reverse primer, 5′-ACCCACACTTCGTTTACCGG-3′ (start position 4126, within the intron); and reverse primer, 5′-GCTTCATCTGCTTCC-3′ (within the intron). Samples were heated at 100°C for 5 min and processed by immunoblots as described above. E2 polymorphism was determined by Southern blotting of genomic DNA from various cell lines treated with E2 as described above. DNA samples were incubating 10 μg of DNA with 1 μl of ERα and 1 μl of anti-hemagglutinin or anti-calmodulin antibodies, respectively. The concentration and incubation times are indicated in the figure legends. Note that the concentration of BPTA-AM used did not reduce the viability of the cell lines used in this study (data not shown). In addition, E2 did not enhance the signal in cells transfected with the TK-Luc plasmid lacking ERE (data not shown).
cDNA sample prepared from MCF-7 cells or COS-7 cells were used to construct standards curves for the pS2 hnRNA amplifications. In all cases, concurrent analysis was performed with β-actin as an internal control. All samples and standards were run in triplicate. Results were analyzed using the relative standard curve method as described in Relative Quantitation of Gene Expression (Applied Biosystems User Bulletin 2). pS2 results were expressed relative to β-actin as internal control.

Miscellaneous—Statistical analysis was performed by Students's t test, using InStat software (GraphPad Software, Inc.). Protein concentrations were determined with the DC Protein Assay (Bio-Rad).

RESULTS

Develop ERα Mutants That Do Not Bind to Calmodulin—We previously reported that ERα interacted directly with calmodulin and that antagonism of calmodulin attenuated ERα transcription (9, 15). To further elucidate the role of calmodulin in ERα function, we set out to develop ERα mutant constructs that would not bind calmodulin. The first step was to identify the calmodulin binding region in ERα. The strategy adopted was to generate radiolabeled fragments of ERα and examine their ability to bind to calmodulin-Sepharose (Fig. 1). [35S]Methionine-labeled full-length ERα (ERα-1–595) bound to calmodulin-Sepharose (Fig. 1B). Specificity of binding to calmodulin is evident by the absence of an interaction with Sepharose alone. Binding was markedly reduced when Ca^{2+} was chelated with EGTA (data not shown). Analysis of fragments of ERα revealed that the middle portion of the molecule was required for maximal binding (Fig. 1). Longer exposure of the autoradiogram in Fig. 1B revealed a weak band in the lane containing ERα-1–270 (data not shown), implying a possible low affinity binding site in this region of the molecule. By generating multiple fragments of different sizes, residues 248–317 were observed to be necessary for maximal binding (Fig. 1C). Inspection with a helical wheel projection of the sequence of ERα in this region revealed a predicted calmodulin binding motif between amino acids 298 and 317 (IKRSKKNLALS/LTADQMVS).

The effect of deletion of residues 298–317 of ERα on its ability to bind calmodulin was examined next. COS-7 cells were transfected with ERα, and lysates were incubated with calmodulin-Sepharose in the presence of Ca^{2+}. Full-length ERα bound to calmodulin (Fig. 2A). Binding to calmodulin was specific as no ERα was detected in samples incubated with Sepharose alone. Deletion of residues 298–317 of ERα eliminated its ability to bind calmodulin (Fig. 2A). Similarly, deletion of smaller portions of ERα, namely amino acids 298–310 and 298–303, abrogated binding to calmodulin. Based on our knowledge of the interaction of calmodulin with target proteins (22), we hypothesized that Ile-298 and Lys-299 were necessary for the interaction of calmodulin with calmodulin. Consistent with our prediction, an ERα point mutant in which Ile-298 and Lys-299 were replaced, termed ERαΔCaM, failed to bind to calmodulin (Fig. 2A).

To verify the findings, calmodulin binding assays were also performed with [35S]methionine-labeled ERα. Wild-type ERα bound to calmodulin in the presence of Ca^{2+}; minimal ERα was detected when Ca^{2+} was chelated with EGTA (Fig. 2B). As was observed with transfected cells, neither radiolabeled ERαΔ298–317 nor ERαΔCaM was able to bind calmodulin. Note that equal amounts of [35S]methionine-labeled ERα were used (Fig. 2B, input).

Comparison of Transcriptional Activities of Wild-type and Mutant ERα—The effects of calmodulin on ERα transcriptional activation were examined by comparing wild-type and mutant ERαs in a well characterized assay that measures the activity of an ERα-responsive reporter (9). To eliminate interference from endogenous ERα, we used COS-7 cells that lack ERα. Incubation with E_{2} enhanced ERα transcriptional activity 5.2-fold in COS-7 cells transiently transfected with wild-type ERα (Fig. 3A). In contrast, transcriptional activity of ERαΔ298–317, which lacks amino acids 298–317, was not significantly stimulated by E_{2}. It is possible that the removal of 20 amino acids may produce a large conformational change in ERα, rendering it transcriptionally inactive. Therefore, analysis was repeated using ERαΔ298–303, which lacks only six amino acids. Although basal transcriptional activity of ERαΔ298–303 was -2-fold higher than that of wild-type ERα, E_{2} was unable to augment transcription (Fig. 3A). Similarly, E_{2} had no effect on the transcriptional activity of the point mutant construct ERαΔCaM. Absent detailed structural information, it is impossible to completely exclude that the conformation of ERαΔCaM is altered. However, it is most unlikely that changing only two amino acids would sufficiently perturb the tertiary structure to prevent ERα binding to transcription factors. These data con-
firm that E₂ cannot stimulate transcriptional activation of 
ERα, which lack the ability to bind calmodulin.

It is conceivable that lack of E₂ stimulation of transcription of the mutant ERα could be due to altered receptor expression. Therefore, an aliquot of the cell lysate was removed prior to the transcription assay and the amount of ERα was evaluated by Western blotting. Mutant ERα was expressed to the same level among all the samples (Fig. 3B). Consistent with published observations in breast epithelial cells by this group (15) and others (23, 24), E₂ reduced the amount of wild-type ERα in COS-7 cells (Fig. 3B). Interestingly incubation with E₂ did not substantially alter the amount of mutant ERα. The mechanism responsible for this finding is not known.

**E₂ Binding**—The calmodulin binding region overlaps the proximal portion of the ligand binding domain (see Fig. 1A). It is therefore feasible that the inability of E₂ to stimulate transcriptional activation of the mutant receptors may be due to an inability of these receptors to bind to E₂. This possibility was addressed by examining binding of [³²P]estradiol to ERα with a whole cell ligand binding assay. Transfecting COS-7 cells with wild-type ERα yielded robust binding of [³²P]estradiol (Fig. 4). (Specific binding was 10-fold greater than nonspecific binding.) In contrast, ERαΔ298–317 completely failed to bind E₂. The findings with ERαΔ298–303 and ERαΔCaM were completely different; the E₂ binding capacity of these ERα mutants was 3.5-fold greater than that of wild-type ERα (Fig. 4). These data validate that ERα298–303 and ERαΔCaM can bind E₂, and the lack of transcriptional activation is not because of an inability to bind E₂.

**Ca²⁺ is Required for E₂-stimulated Transcriptional Activity**—We (15) and others (25, 26) have documented that Ca²⁺ is necessary for the interaction between calmodulin and ERα. Therefore, on the basis of the data in Fig. 3, one would anticipate that chelation of [Ca²⁺] would reduce the ability of E₂ to stimulate transcription by ERα. This hypothesis was tested by incubating MCF-7 cells with the cell-permeable Ca²⁺ chelator BAPTA-AM. Chelation of intracellular Ca²⁺ abrogated E₂-stimulated ERα transcriptional activity (Fig. 5A). BAPTA, which did not significantly alter cell viability under our assay conditions, had no significant effect on basal ERα transcription. In addition, Western blotting demonstrated that BAPTA did not substantially change the amount of ERα in the cells (Fig. 5B). These data are consistent with a requirement of Ca²⁺/calmodulin binding for ERα transcriptional activity.

**Effect on ERα Transcriptional Activity of a Mutant Calmodulin Unable to Bind Ca²⁺**—Calmodulin contains four Ca²⁺ binding domains, two in the N-terminal-half and two in the C-terminal-half of the molecule (27). The identification of the specific amino acids in calmodulin that bind Ca²⁺ has permitted the development of point mutant calmodulin constructs that are unable to bind Ca²⁺ (17, 28–30). These mutants have been shown to function as dominant negative calmodulin constructs in some situations (30, 31). To examine a possible effect on ERα transcription, CalM1234 was transfected into MCF-7 cells and ERα transcriptional activity was measured. Transient transfection of CalM1234 reduced by 50% basal ERα transcriptional activity (Fig. 6A). In addition, E₂ was unable to significantly enhance ERα transcriptional activity in cells transfected with CalM1234 (Fig. 6). Consistent with our prior documenta-
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FIG. 4. E2 binding capacity of full-length and mutant ERα. COS-7 cells were transiently transfected with full-length ERα (WT), ERαΔ298–317, ERαΔ298–303, or ERαΔCaM. Twenty-four hours later the culture medium was replaced with phenol red-free medium for another 24 h, followed by 2 h of incubation with 10 nM [3H]estradiol. Cells were lysed, and the amount of bound radioactivity was quantified by liquid scintillation spectrophotometry. Results are expressed relative to cells treated with vehicle (clear bars) or 10 nM E2 (shaded bars). After incubating for 16 h, samples were assayed for luciferase activity. Results are expressed relative to cells treated with vehicle alone, which was set as 1.0. * significantly different (p < 0.05); ** significantly different (p < 0.001). Data are the means of three separate experiments, each performed in duplicate. Means ± S.E. are shown. The absence of error bars from ERαΔ298–317 indicates that the range is too small to be visible.

FIG. 5. Chelation of [Ca2+]i inhibited E2-induced ERα transcriptional activity. A, MCF-7 cells were transiently co-transfected with ERES-TK-Luc and pRL-TK. Vehicle (DMSO) or 20 μM BAPTA was added, followed immediately with vehicle (clear bars) or 10 nM E2 (shaded bars). After incubating for 16 h, samples were assayed for luciferase activity. Results are expressed relative to cells treated with vehicle alone, which was set as 1.0. * significantly different (p < 0.05); † not significant (p > 0.05). Data are the means of three separate experiments, each performed in duplicate. Means ± S.E. are shown. B, equal amounts of protein lysate from the cells treated as described in A were removed and processed by SDS-PAGE. After transfer to PVDF, blots were probed with anti-ERα antibody, and antigen-antibody complexes were identified by ECL. A representative experiment is shown.

The effects of Ca2+ on the ability to promote transcriptional activation of pS2 was also confirmed by quantitative RT-PCR. pS2 expression is accepted as a reliable measure of transcriptional activity of ERα (21, 32, 33).

When COS-7 cells were transfected with wild-type ERα, E2 significantly increased the transcriptional activity of the pS2 gene (Fig. 7A). In contrast, in cells transfected with an equivalent amount of ERαCaM the ability of E2 to increase pS2 hnRNA was dramatically reduced. Thus, the elimination of calmodulin binding prevented ERα from increasing transcription of two independent reporters in response to E2.

The effects of chelating [Ca2+]i were evaluated in the same assay. Analogous to the results with ERE, BAPTA eliminated E2-induced transcriptional activation of pS2 in MCF-7 cells (Fig. 7B). Finally, transcription of pS2 was analyzed in the presence of CaM1234. Transfection of MCF-7 cells with native calmodulin did not substantially alter E2-induced expression of pS2 hnRNA (Fig. 7C). In contrast, CaM1234 reduced by 33% the ability of E2 to promote transcriptional activation of pS2 hnRNA by endogenous ERα (Fig. 7C). The magnitude of the reduction of pS2 hnRNA activation produced by CaM1234 was very similar to the magnitude of the inhibition CaM1234 produced on E2-stimulated ERE transcription (see Fig. 6). Collectively, the data obtained with pS2 using three complementary strategies, namely ERαCaM, BAPTA and CaM1234, validate the findings generated with ERE that calmodulin is important for E2-stimulated transcriptional activity of ERα.

DISCUSSION

A fairly large body of published literature implicates Ca2+ and calmodulin in ERα function (Ref. 15 and references therein). Notwithstanding this evidence, relatively little attention is focused on the role of calmodulin in ERα signal transduction. Several factors are likely to account for this situation. Probably one of the most important reasons is that the majority of evidence implicating Ca2+ and calmodulin in ERα function has been derived with chemical inhibitors of calmodulin and is therefore indirect. In order to address this deficiency, we combined the use of mutant and dominant negative constructs to validate that calmodulin is necessary for normal transcriptional function of ERα.

The initial strategy was to generate mutant ERα that is unable to bind calmodulin. Although lacking sequence homology, calmodulin binding domains on target proteins generally fall into one of two main groups: (i) short regions (14–26 amino acid residues) that form basic amphiphilic α-helices (34) and (ii) the IQ motif, which has a consensus sequence...
IQXXRgGXXX (where X is any amino acid) (22, 35). No IQ motif was detected in E\(\alpha\) and the sequence (corresponding to amino acids 298–317 in human E\(\alpha\)) to which calmodulin binds does not correspond to a canonical target motif (35). These findings are not surprising as recent evidence reveals considerable diversity and variability in the interaction of calmodulin with its target molecules (36). Our data indicate that amino acid residues 298–303 of E\(\alpha\) are necessary for calmodulin binding, with Ile-298 and Lys-299 appearing to be essential.

The calmodulin binding domain in E\(\alpha\) is close to the middle of the receptor, located at the N-terminal portion of the hormone binding domain and extends partially into the hinge region. This location has potentially important consequences for E\(\alpha\) function. In fact, analysis of transcriptional function revealed that all the E\(\alpha\) mutant constructs that cannot bind calmodulin failed to increase transcription in response to E\(_2\). In order to minimize the possible disruption of E\(\alpha\) structure produced by deletion of amino acids, we generated a point mutant construct that is unable to bind calmodulin. Changing only two amino acids is highly unlikely to produce a dramatic change in conformation. Like the deletion mutants, E\(\alpha\)ΔCaM was unable to increase transcription when stimulated with E\(_2\). These data provide direct evidence to validate our prior observations (obtained with calmodulin inhibitors) (9) that calmodulin has a role in E\(_2\)-stimulated transcriptional activity of E\(\alpha\). Nevertheless, some caveats of our work should be borne in mind. Binding of ligand to E\(\alpha\) induces conformational changes in E\(\alpha\), resulting in enhanced transcription. Calmodulin binds in the hinge region of E\(\alpha\), immediately proximal to the ligand binding domain. Therefore, it is conceivable that the two amino acid substitutions could modify secondary or tertiary structure of E\(\alpha\). Should this occur, E\(\alpha\) dimersization, DNA binding and/or interaction with other targets might be altered independent of calmodulin binding. This premise can be eliminated only by solving the structure of the mutant E\(\alpha\).

Our data differ from a prior report describing a mutant E\(\alpha\) deficient in calmodulin binding (10). In that work, a point mutant E\(\alpha\), termed E\(\alpha\) (K302G, K303G) (the lysines at 302 and 303 were replaced with glycine), had the same basal and E\(_2\)-stimulated transcriptional activity as wild-type E\(\alpha\). Several factors might contribute to the disparate results between the studies. Although exhibiting reduced affinity for calmodulin, E\(\alpha\) (K302G, K303G) is able to bind \(-20\%\) as much calmodulin as wild-type E\(\alpha\). In contrast, E\(\alpha\)ΔCaM used in our study bound no detectable calmodulin. In addition, we used different reporter constructs and cell lines to those employed in the prior work. Another potentially important factor to account for the findings obtained with E\(\alpha\) (K302G, K303G) by Ramos’s group is that Lys-302 and Lys-303 of E\(\alpha\) are targets for acetylation by p300 (37). In fact, Wang et al. (37) observed that E\(\alpha\) mutated at Lys-302 and Lys-303 exhibited E\(_2\)-stimulated transcriptional activity that was 2–4-fold higher than that of wild-type E\(\alpha\). Consistent with the latter study, other groups observed that mutation of Lys-303 resulted in hypersensitivity to E\(_2\) in E\(\alpha\) transactivation assays (38, 39). The reason for the discrepancy between the reports that examined the Lys-303 E\(\alpha\) mutants is not clear. These factors, particularly the retention of some calmodulin binding and elimination of E\(\alpha\) acetylation, confound interpretation of the role of calmodulin in E\(\alpha\) function in the study by Ramos’s group. Importantly, the point mutations introduced into E\(\alpha\)ΔCaM used in our study abrogated calmodulin binding. Moreover, to the best of our knowledge, no post-translational modifications have been identified for the residues we mutated, namely Ile-298 or Lys-299. Therefore, we believe that our investigation for the first time directly assesses the role of calmodulin in E\(\alpha\) transcription.

Unexpectedly, some E\(\alpha\) constructs deficient in calmodulin binding exhibited enhanced basal transcriptional activity. While the cause of this effect has not been identified, a possible clue is provided by the E\(_2\) binding analyses: E\(\alpha\)ΔCaM bound 3.5-fold more E\(_2\) than wild-type E\(\alpha\). Although the molecular mechanism responsible for the enhanced binding is not known, these findings are consistent with the observation that calmodulin mediates a decrease in E\(_2\) binding to E\(\alpha\) (11). The calmodulin binding domain is immediately proximal to (and may even partially overlap) the E\(_2\) binding site, suggesting that
Calmodulin may sterically hinder E2 binding. As we previously hypothesized (15), it is likely that direct binding to calmodulin alters the tertiary conformation of ERα/H9251. Absent calmodulin binding, the receptor may adopt a conformation that permits increased E2 binding. Solving the structure of ERα/H9251/CaM is necessary to provide a definite answer.

Two independent approaches bolster our observations on transcription derived with the mutant ERα/H9251 constructs. [Ca2+]i was chelated with BAPTA (40). Cells were loaded with BAPTA-AM, the acetoxymethyl ester of BAPTA. This derivative is non-polar and permeates into cells where endogenous esterases remove the ester groups, leaving the membrane-impermeable chelator trapped in the cell. BAPTA has been widely used to investigate intracellular Ca2+ function (40–42). However, caution should always be exercised when interpreting results obtained in cells with chemical compounds. For example, chelation of [Ca2+]i can produce effects in the cell that are independent of calmodulin. Notwithstanding this caveat, Ca2+ is necessary for the interaction between calmodulin and ERα (15, 25, 26) and Fig. 2B, this study). Therefore, one would anticipate that if calmodulin binding is necessary for E2-stimulated transcription, removing [Ca2+]i would eliminate E2-stimulated transcription. That is exactly what we observed, further supporting our hypothesis.

The last strategy employed CaM1234, a mutant calmodulin incapable of binding Ca2+ (17, 30). CaM1234 has a dominant negative effect in some cellular processes (30, 31). Congruent with these observations, CaM1234 was a dominant negative in ERα transcription. CaM1234 reduced E2-stimulated ERα transcriptional activity for both ERE and the endogenous estrogen responsive gene pS2. Because the Ca2+-deficient CaM1234 is not expected to bind ERα, the dominant negative effect is presumably mediated by the Ca2+-independent binding of CaM1234 to (an)other molecule(s) that is (are) a component of

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**FIG. 7. Effects of ERαCaM, BAPTA, and CaM1234 on E2-induced transcriptional activity of the estrogen responsive gene pS2.**

**A.** COS-7 cells were transiently transfected with wild-type ERα (WT) or ERαΔCaM (ΔCaM). Twenty-four hours later the culture medium was replaced with phenol red-free medium for 24 h, followed by a 6-h incubation with vehicle (EtOH, clear bars) or 100 nM E2 (shaded bars). Total RNA was extracted and subjected to quantitative RT-PCR analysis to determine the expression levels of pS2 hnrRNA. For all RT-PCR assays, the relative levels of mRNA were normalized with β-actin mRNA. Values obtained in vehicle-treated cells transfected with wild-type ERα were set to 1. Data represent the means ± S.E. of three independent experiments, each performed in triplicate.

**B.** MCF-7 cells were grown in phenol red-free medium for 24 h. Cells were incubated with vehicle (DMSO) or 20 μM BAPTA, followed immediately with vehicle (clear bars) or 100 nM E2 (shaded bars) for 6 h. Total RNA was prepared and subjected to RT-PCR analysis as described for A. Data are representative of two independent experimental determinations. Error bars depict S.E. derived from triplicate analysis.

**C.** MCF-7 cells were transiently transfected with pcDNA3 vector (V), CaM1234 or native calmodulin (CaM). Twenty-four hours later, cells were incubated with vehicle (EtOH) or 100 nM E2 for 6 h. RT-PCR was performed as described for A. Data are representative of two independent experimental determinations. Error bars depict S.E. derived from triplicate analysis.
the ERα transcriptional apparatus. Regardless of the mechanism, our findings, obtained using diverse, but complementary analytical strategies, reveal that E2-stimulated transcriptional activity of ERα is dependent on Ca²⁺/calmodulin.

The specific role played by calmodulin in E2-stimulated ERα transcription is not known. We previously documented that calmodulin binds to ERα in a Ca²⁺-dependent manner, thereby reducing ERα degradation (15). However, the stabilizing function of calmodulin appears to be independent of its transcriptional effect (9), and thus does not explain the requirement of calmodulin for transcription. The interaction of calmodulin with its targets frequently induces a conformational change in the target protein (43). Therefore, a more probable explanation for the effect of calmodulin on ERα transcription is that the binding of calmodulin alters the tertiary conformation of ERα, modulating the interaction of the receptor with (an)other protein(s). The ability of calmodulin to alter ERα conformation can be inferred from the protection afforded by calmodulin to in vitro proteolysis of ERα (15). Considerable effort over the past few years has been directed toward the identification and characterization of adaptor proteins that modulate ERα function. These targets include coactivators and corepressors (3). Recent evidence reveals that calmodulin is an integral component of an ERα-ERE complex (12). Because calmodulin was necessary, but not sufficient, for complex formation, auxiliary proteins are believed to participate (12). Therefore, calmodulin is likely to modulate ERα function by altering the binding of ERα to transcriptional coactivators or corepressors. Collectively, these data suggest that the molecular interaction between calmodulin and ERα could be the target for therapeutic intervention in patients with breast cancer.

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REFERENCES
1. Hall, J. M., Couse, J. F., and Korach, K. S. (2001) J. Biol. Chem. 276, 36869–36872
2. Dickson, R. B., and Stancel, G. M. (2000) J. Natl. Cancer Inst. Monogr. 27, 135–145
3. McKenna, N. J., Lanz, R. B., and O'Malley, B. W. (1999) Endocr. Rev. 20, 321–344
4. Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M., and Gustafsson, J. A. (2001) Physiol. Rev. 81, 1535–1565
5. McDonnell, D. P., and Norris, J. D. (2002) Science 296, 1642–1644
6. Levin, E. R. (2002) Steroids 67, 471–475
7. Meyer, M. E., Gronemeyer, H., Turcotte, B., Bocquel, M. T., Tasset, D., and Chambon, P. (1989) Cell 57, 433–442
8. Trumeter, M. T., Eddy, A., Santiso-Mere, D., Danielian, P., Parker, M. G., Stein, R. B., Pike, J. W., and McDonnell, D. P. (1994) Mol. Endocrinol. 8, 21–39
9. Li, L., Li, Z., and Sacks, D. B. (2003) J. Biol. Chem. 278, 1195–1200
10. Garcia-Pedraja, J. M., Del Rio, B., Martinez-Campa, C., Muramatsu, M., Lazo, P. S., and Ramos, S. (2002) Mol. Endocrinol. 16, 947–960
11. Bouhoute, A., and Leclercq, G. (1995) Biochem. Biophys. Res. Commun. 208, 468–475
12. Biswas, D. K., Reddy, P. V., Pickard, M., Makkad, B., Petit, N., and Pardee, A. B. (1998) J. Biol. Chem. 273, 33817–33824
13. Pinzane, J. J., Stevenson, H., Strobl, J. S., and Berg, P. E. (2004) Mol. Cell. Biol. 24, 4605–4612
14. Weihua, Z., Andersson, S., Cheng, G., Simpson, E. R., Warner, M., and Gustafsson, J. A. (2003) FEBS Lett. 546, 17–24
15. Li, Z., Joyal, J. L., and Sacks, D. B. (2001) J. Biol. Chem. 276, 17354–17360
16. Sacks, D. B., Porter, S. E., Ladenson, J. H., and McDonald, J. M. (1991) Anal. Biochem. 194, 369–377
17. Peterson, B. Z., DeMaria, C. D., Adelman, J. J., and Yue, D. T. (1999) Neuron 22, 549–558
18. Briggs, M. W., Li, Z., and Sacks, D. B. (2002) J. Biol. Chem. 277, 7453–7465
19. Roy, M., Li, Z., and Sacks, D. B. (2004) J. Biol. Chem. 279, 17329–17337
20. Reese, J. C., and Katzenellenbogen, B. S. (1991) J. Biol. Chem. 266, 10860–10867
21. Fan, M., Nakshatri, H., and Nephew, K. P. (2004) Mol. Endocrinol. 18, 2603–2615
22. Li, Z., and Sacks, D. B. (2003) J. Biol. Chem. 278, 4347–4352
23. Berthoix, Y., Dong, X. F., Roux-Dussette, M., and Martin, P. M. (1990) Mol. Cell. Endocrinol. 74, 11–20
24. Nawaz, Z., Lonard, D. M., Dennis, A. P., Smith, C. L., and O'Malley, B. W. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1854–1862
25. Bouhoute, A., and Leclercq, G. (1994) Biochem. Pharmacol. 47, 748–751
26. Castoria, G., Migliaccio, A., Nola, E., and Auricchio, F. (1988) Mol. Endocrinol. 2, 167–174
27. Babu, Y. S., Sack, J. S., Greenhough, T. J., Bugg, C. E., Means, A. R., and Cook, W. J. (1985) Nature 315, 37–40
28. Maune, J. F., Klee, C. B., and Beckham, K. (1992) J. Biol. Chem. 267, 5286–5295
29. Lee, W. S., Ngo-Anh, T. J., Bruning-Wright, A., Maylie, J., and Adelman, J. P. (2003) J. Biol. Chem. 278, 25940–25946
30. DeMaria, C. D., Song, T. W., Abelskahan, B. A., Alvania, R. S., and Yue, D. T. (2001) Nature 411, 484–489
31. Zuhlike, R. D., Pitt, G. S., Deisseroth, K., Tsien, R. W., and Reuter, H. (1999) Nature 399, 159–162
32. Brown, A. M., Jeloch, J. M., Roberts, M., and Chambon, P. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 6344–6348
33. Shao, W., Keeton, E. K., McDonnell, D. P., and Brown, M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 11599–11604
34. Crivici, A., and Ikura, M. (1995) Annu. Rev. Biophys. Biomol. Struct. 24, 85–116
35. Bahler, M., and Rhoads, A. (2002) FEBS Lett. 513, 107–113
36. Hoeflich, K. P., and Ikura, M. (2002) Cell 108, 739–742
37. Wang, C., Fu, M., Angeletti, R. H., Stizolfi-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., and Pestell, R. G. (2001) J. Biol. Chem. 276, 16375–16383
38. Mishra, S. K., Mazumdar, A., Vadlamudi, R. K., Li, F., Wang, R. A., Yu, W., Jordan, V. C., Santen, R. J., and Kumar, R. (2003) J. Biol. Chem. 278, 19209–19219
39. Cui, Y., Zhang, M., Pestell, R., Curran, E. M., Welshons, W. V., and Fuqua, S. A. (2004) Cancer Res. 64, 9199–9208
40. Metcalfe, J. C., and Smith, G. A. (1991) in Cellular Calcium (McCormack, J. G., and Cobbold, P. H., eds) pp. 123–132, Oxford University Press, New York
41. Bouchard, M. J., Wang, L. H., and Schneider, R. J. (2001) Science 294, 2376–2378
42. Pusi, T., Wu, J. J., Zimmerman, T. L., Zhang, L., Ehrlich, B. E., Berchtold, M. W., Hoek, J. B., Karpen, S. J., Nathanson, M. H., and Bennett, A. M. (2002) J. Biol. Chem. 277, 27517–27527
43. Cohen, P., and Klee, C. (1988) Calmodulin, Elsevier, New York