Calmodulin (CaM) has been reported to have affinity for the estrogen receptor (ER). Observations reported here reveal a direct physical interaction between purified CaM and ER. This direct ER-CaM interaction may be an initial event preceding the assembly of ER plus auxiliary proteins into the active ER complex with its DNA motif, the estrogen response element. We demonstrate that CaM is an integral component of this complex by using a system reconstituted from purified ER and nuclear extract from ER-negative breast cancer cells and also with ER-depleted nuclear extract of an ER-positive breast cancer cell line. Although CaM is essential for formation of this complex, it is not sufficient, suggesting roles also of auxiliary proteins. CaM also is functionally required for activation of an ER-responsive promoter, in the 17β-estradiol-ER pathway of hormone action and regulation of 17β-estradiol-responsive gene expression that is associated with proliferation of mammary epithelial cells.

CaM\(^1\) plays a pivotal role in the proliferation of a variety of cells. Several observations indicate CaM involvement in estrogen regulation of breast cancer cell growth. Calcium homeostasis is lost (1), and expression of calcium binding proteins is modulated (2–4). There is an overall increase of Ca\(^{2+}\) levels in human mammary tumors (5, 6), and CaM concentrations are 2–3-fold higher in estrogen receptor-positive (ER\(^+\)) than in estrogen receptor-negative (ER\(^–\)) breast tumors (7). The growth of breast cancer cells is highly sensitive to anti-calmodulin drugs (8, 9), and the anti-estrogen Tamoxifen binds to CaM with high affinity and antagonizes its action (10). CaM also modulates estrogen (17β-estradiol (E2)) binding to ER (11), and synergistic inhibition of growth by CaM inhibitors and antiestrogens (12, 13) are associated with breast cancer.

Both anti-estrogens and anti-calmodulin drugs block breast cancer cells at an identical point in the G1 phase of the cell cycle (14). Furthermore, CaM is known to stimulate the phosphorylation of estrogen receptors (15). Biochemical evidence suggests an interaction of CaM with ER (11, 16–19), but these results do not demonstrate a direct physical contact.

We examined more directly the possibility of a role of CaM in key downstream events of E2 action, i.e. the interaction of ER protein with its cognate DNA sequence (estrogen response element (ERE)) to form the ERE-ERE complex and the resulting transactivation of an E2-responsive gene. We observed that CaM directly interacts with ER, and is not only an integral component of the ERE-ERE complex but also plays an essential role in complex formation. Its functional involvement in the molecular pathway of E2-induced transactivation of hormone-responsive genes in breast cancers was tested.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture**—MCF-7 and MDA-MB-231 cell lines were obtained from ATCC and were grown in rich medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2.8 µg hydrocortisone, 1 µg/ml insulin, and 12.5 ng/ml epidermal growth factor) under standard tissue culture conditions (20, 21). For hormone studies Dulbecco's modified Eagle's medium without phenol red (DCC) supplemented with 10% stripped serum (dextran-coated, charcoal-treated fetal bovine serum; HyClone) was used.

**Materials**—Synthetic single-stranded complementary oligonucleotides containing a wild-type ERE motif (5'-GTTCAGGGTGTTCA-CAGTGACCCTGTCAAGTTG-3') were obtained from Life Technologies, Inc. These strands were annealed to generate the double-stranded ERE oligonucleotide. E2, hydrocortisone, insulin, dithiothreitol, dimethyl sulfoxide, phenylmethylsulfonyl fluoride, CaM, anti-calmodulin antibody, and (N-6-aminohexyl)-5-chloro-1-naphthalene sulfonamide hydrochloride (W7) were from Sigma. SuperFect was from Qiagen. Monoclonal anti-ER antibody TE111, raised against C-terminal amino acid residues 300–595 of human ER, was obtained from NeoMarkers (Fremont, CA). Purified human recombinant ER (22) was a gift from Fan Vera Corp. (Madison, WI). The ECL immunodetection kit was from Amersham Pharmacia Biotech.

**Reconstitution and Identification of ERE-ERE Complex by Electrophoretic Mobility Shift Assay (EMSA)**—The ER used was purified from recombinant baculovirus-infected insect Sf9 cells by Pan Vera by a modified method (22). Nuclear extract from MDA-MB-231 cells was prepared according to the method of Dignam et al. (23). Indicated amounts (see figure legends) of purified ER and nuclear extracts were preincubated in binding buffer (24) for 15 min at room temperature. The reaction mixture was then incubated for an additional 30 min at room temperature in the presence of 1 ng of \(^{32}\)P-labeled (30,000 cpm) double-stranded synthetic oligonucleotides with palindromic ERE (shown above). The reaction mixture was then subjected to EMSA, and the protein-DNA complex was detected as a retarded radioactive band by autoradiography of the dried gel as described (25, 26). Supershift assays were performed by preincubating the reaction mixture containing pure ER, nuclear extracts from MDA-MB-231 cells, and the specified antibody (see figure legends) in binding buffer for 15 min at room temperature followed by incubation with \(^{32}\)P-ERE oligonucleotide for an additional 30 min at room temperature. The reaction mixture was...
then analyzed by EMSA, and the supershifted ER-ERE complex was detected by autoradiography. The intensity of the retarded radioactive band was quantitated by scanning with a densitometer (Bio-Rad GS-700), and the intensity of the bands was integrated by using the Molecular Dynamics program, version 1.0.2. The intensities was determined using the Molecular Dynamics program, version 1.0.2, and presented as % band intensity.

Immunodepletion of Nuclear Extracts—Nuclear extracts from MDA-MB-231 and MCF-7 cells were incubated with either anti-ER antibody or anti-CaM antibody for 1 h at room temperature with gentle rocking. Protein A/G-agarose beads in 3× excess of the specific antibody were then added, and the mixture was left on the rocker in the cold room for an additional hour. The protein A/G-agarose-antibody-antigen complex was then removed by centrifugation at 2500 rpm for 5 min in a microcentrifuge. The supernatant was saved (depleted extract), and the level of the specific antigen in the supernatant, either ER or CaM, was determined by Western blot analysis. The ER- or CaM-depleted nuclear extracts were then used for reconstitution studies by EMSA.

Identification of Co-immunoprecipitated ER and Calmodulin in the ER-ERE Complex—The precipitation reaction was performed with purified ER and nuclear extract of MDA-MB-231 cells, as described above, in the presence of appropriate amounts of respective antibody as indicated in figure legends, followed by incubation in the absence or presence of a 1000-fold excess of nonradioactive, double-stranded ERE oligonucleotide. The antibody-antigen complex was then incubated with protein A/G-agarose beads on a rocker at 4 °C for 1 h, followed by centrifugation at 2500 rpm for 5 min. The supernatant was discarded, and the precipitated antigen-antibody complex was washed three times with binding buffer. The pellet was then resuspended in 20 μl of Laemmli buffer (27), boiled for 5 min, cooled to room temperature, and centrifuged, and the supernatant was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by electroblotting to a polyvinylidene difluoride membrane. Immunoblot analysis was performed with a mouse anti-human ER-antibody or mouse anti-human CaM antibody followed by incubation with goat anti-mouse secondary IgG-horseradish peroxidase conjugate and was immunodetected with an ECL system (Amersham Pharmacia Biotech).

Direct Interaction of ER and CaM under Nondenaturing and Denaturing Conditions—Direct interaction of human recombinant ER with purified CaM under nondenaturing conditions was studied by incubation of indicated amounts of ER and CaM (see figure legends) under binding conditions described above in 1× binding buffer (24). The binding reactions were performed in the absence of nuclear proteins and in the presence of indicated components. The reaction samples were then subjected to 6% PAGE analysis under nondenaturing conditions followed by Western blot transfer onto a nitrocellulose membrane (Hybond, Amersham Pharmacia Biotech) in buffer without methanol. Separate membranes were then probed with anti-ER antibody and anti-CaM antibody. ER, CaM, and ER-CaM complexes were then detected by the ECL immunodetection system as described above.

Direct interaction of ER and CaM was also studied under denaturing conditions. The reaction tubes containing pure human recombinant ER and CaM were incubated at room temperature for 30 min, immunoprecipitated with either anti-ER antibody or anti-CaM antibody, and subjected to SDS-PAGE, blot-transferred, processed under conditions described above, and immunodetected by using the indicated antibody and enhanced chemiluminescence system.

Transfection of MCF-7 Cells with pPS2-CAT—MCF-7 cells were grown in rich medium for 24 h followed by growth in fresh DCC medium supplemented with 10% stripped fetal bovine serum for an additional 72 h. Cells were then transfected with the fusion plasmid pPS2-CAT (28) using SuperFect following the protocol provided by the supplier and 48 h later were treated with indicated amounts of E2 with or without W7 for 2 h and harvested; cell extracts were made, chloramphenicol acetyltransferase (CAT) activity in equal amounts of protein (29) was measured as described (25, 26), and activity was quantitated as described in the legend to Fig. 7.

RESULTS

CaM Is an Integral Component of the ER-ERE Complex—We have studied ER-ERE complex formation in a reconstituted system using purified recombinant ER (22) and nuclear extract (23) from the ER− breast cancer cell line MDA-MB-231. Purified ER formed a complex with 32P-labeled ERE oligonucleotide only in the presence of nuclear extract from MDA-MB-231 cells (Fig. 1, A, lane 3, and B, lane 5; each lane is represented by duplicate samples), suggesting that auxiliary proteins are necessary for ER-ERE complex formation. Substitution of nuclear extract with equal amounts of nonspecific proteins such as bovine serum albumin (BSA) did not form the ER-ERE complex under these reconstitution conditions (Fig. 1A, lane 1). Nuclear extract from MDA-MB-231 cells that are deficient in ER did not by itself form a complex with 32P-labeled ERE oligonucleotide (Fig. 1A, lane 2).

Presence of ER in this retarded complex was confirmed by its interaction with a monoclonal anti-ER antibody that caused a supershift of the protein-DNA complex in EMSA analysis (Fig. 1A, lane 4). A control mouse IgG under similar experimental conditions failed to produce any supershift of ER-ERE complex (data not shown).

We similarly identified CaM in the ER-ERE complex by preincubating the reaction mixture in the presence of monoclonal anti-CaM antibody, which supershifted the reconstituted complex (Fig. 1B, lane 6). Again the same amounts of mouse IgG did not cause supershift of the ER-ERE.

Binding of ER to 32P-ERE-containing oligonucleotide was detected in the nuclear extract prepared from cells grown in E2-free medium in the absence of E2, suggesting that ER-ERE interaction in this reconstitution system is independent of E2, as has been observed by other investigators (30). Stimulation by E2 of ER-32P-ERE binding was detected when hormone-treated, ER+ MCF-7 cells (with native ER) were used to prepare nuclear extracts (Fig. 2A, lane 2). However, addition of E2 directly to the reaction mixture containing nuclear extracts...
from untreated MCF-7 cells was not stimulatory (Fig. 2A, lane 3).

EGTA treated MDA-MB-231 nuclear extract was deficient in reconstituting the ER-ERE complex, suggesting that assembly is calcium-dependent; presumably calcium is required for binding to CaM (Fig. 2B). Inhibition of 32P-ERE-ERE complex formation by EGTA and the supershift induced by anti-CaM antibody suggest that CaM is an integral component of the ER-ERE complex and represents one of the auxiliary proteins in the nuclear extract of MDA-MB-231 cells that is required for assembly of the complex.

Depletion of Nuclear Extracts with Anti-ER and Anti-CaM Monoclonal Antibodies—An additional experimental approach involves CaM depletion of nuclear extracts. The nuclear extract of ER+ MCF-7 cells was immunodepleted of ER or CaM by anti-ER or anti-CaM antibody, respectively, as described under “Experimental Procedures.” An equal amount of each of the immunodepleted extracts was then examined for the presence of both ER and CaM by Western blot analysis. As shown in Fig. 3A, the ER in extracts immunodepleted by using specific monoclonal antibodies against either ER (lane 3) or CaM (lane 4) was reduced significantly in comparison with extracts undepleted (lane 1) or mock-depleted with mouse IgG (lane 2). A quantitative analysis of average ER band intensities of three separate experiments like the one shown in Fig. 3A is presented in Fig. 3B. This observation that ER could be removed from the nuclear extract by anti-CaM antibody treatment again demonstrates that ER and CaM exist as a complex in the nuclear extract. This is corroborated by the observation that immunodepletion of MCF-7 cell nuclear extract with ER-specific monoclonal antibody caused a dramatic decrease of the level of CaM in these extracts (Fig. 3C, compare lanes 6 and 4). As expected, immunodepletion with anti-CaM antibody resulted in a significantly reduced level of CaM in the nuclear extracts of both ER+ MCF-7 (Fig. 3C, compare lanes 5 and 4) and ER– MDA-MB-231 (Fig. 3C, compare lanes 2 and 1) cells. Intensities of CaM in bands of Fig. 3C are expressed quantitatively in Fig. 3D.

It is important to note that the monoclonal antibodies used for immunodepletion in these studies are highly specific, with ER antibody binding to ER but not to CaM (Fig. 3A, lane 1) and CaM antibody binding to CaM and not to ER (Fig. 3C, lanes 1 and 4), as determined by Western blot analysis. Thus, in these immunodepletion studies, ER antibody probably removed CaM by virtue of its interaction with ER, and similarly CaM antibody removed ER by virtue of its interaction with CaM.

Co-immunoprecipitation of ER and CaM in the Reconstituted ER-ERE Complex—CaM association with ER in the ER-ERE complex is further substantiated by the observation that both CaM and ER could be detected in immunoprecipitates prepared using monoclonal antibodies against either CaM or ER. As shown in Fig. 4A, when ER-ERE reconstitution samples were immunoprecipitated with anti-ER antibody (Fig. 4A, lanes 1 and 2) or with anti-CaM antibody (Fig. 4A, lanes 3 and 4), ER was detected in both immunoprecipitated complexes using Western blot analysis with anti-ER antibody. Similarly, CaM protein was detected in both anti-ER (Fig. 4B, lanes 1 and 2) and anti-CaM (Fig. 4B, lanes 3 and 4) antibody-immunoprecipitated complexes by Western blot analysis with anti-CaM antibody. By comparison, neither ER (Fig. 4A, lanes 5 and 6) nor CaM (Fig. 4B, lanes 5 and 6) was detected after mock immunoprecipitation with mouse IgG.

To examine whether ER and CaM can interact independently of the ERE motif, co-immunoprecipitation reactions were carried out (Fig. 4) in the absence (ERE-Oligo – lanes) or presence (ERE-Oligo + lanes) of ERE-containing oligonucleotide. No major difference was observed between the two reaction conditions, suggesting that ER-CaM complex formation is independent of ERE oligonucleotide. Thus it may be postulated that ER and CaM can interact directly and exist in the nuclear extract as a preformed complex. This is substantiated by the results presented in Fig. 5.

ER and CaM Interact Directly in the Absence of Other Auxiliary Proteins—To examine the possible direct interaction of ER and CaM, we undertook two experimental approaches. First, pure recombinant human ER and pure CaM were incubated together under the reaction conditions for reconstitution of the ER-ERE complex, in the absence of nuclear extracts from MDA-MB-231 cells. The reaction mixtures were then fractionated by polyacrylamide gel electrophoresis under nondenatur-
ing conditions, blot-transferred to nitrocellulose membranes (Hybond, Amersham Pharmacia Biotech), and subjected to Western blot analysis. One membrane was probed with anti-ER antibody (Fig. 5A), and the other was probed with anti-CaM antibody (Fig. 5B). A complex comparatively slower moving than either ER (Fig. 5A, lane 5) or CaM (Fig. 5B, lane 5), presumably ER-CaM, demonstrated direct physical interaction of ER and CaM. This ER-CaM interaction was independent of the presence of auxiliary or nonspecific proteins such as BSA or the ERE oligonucleotide. It was sensitive to EGTA (Fig. 5A and B, lane 6). Addition of E2 to the reaction mixture did not influence ER-CaM interaction (data not shown). The intense and diffuse band identified as ER may be attributable to the large amount (1 pmol) of pure recombinant human ER used, and possibly aggregated pure ER formed under nondenaturing conditions. The stoichiometry of the physical interaction of ER and CaM is not defined as yet, but formation of the ER-CaM complex was more definite at this high concentration of ER.

In the second experimental approach, pure recombinant ER was incubated with CaM under conditions described above, and the reaction mixtures were subjected to immunoprecipitation with either anti-ER antibody or anti-CaM antibody followed by SDS-PAGE and Western blot analysis as described under “Experimental Procedures.” The membrane with samples precipitated was probed with monoclonal anti-ER antibody TE1-11 (A), and the second was probed with monoclonal anti-CaM antibody (B) followed by incubation with the secondary anti-mouse IgG-horseradish peroxidase conjugate. Intensities of the bands were quantitated by densitometric scanning and integration of the values as described under “Experimental Procedures.” IP-Ab, specific antibody used for immunoprecipitation; – and +, absence and presence, respectively, of the specific reagents in the reaction mixture.
tated by anti-CaM antibody was probed with anti-ER antibody (Fig. 5C) and vice versa (Fig. 5D). The band intensities were quantitated by densitometric scanning as described under “Experimental Procedures.” Both ER and CaM were detected in reaction mixtures containing only ER and CaM, in the absence of any auxiliary proteins or nuclear extracts from MDA-MB-231 cells. ER was co-immunoprecipitated with CaM after treatment with anti-CaM antibody (Fig. 5C), and CaM was co-immunoprecipitated with ER after anti-CaM antibody treatment (Fig. 5D). These results again demonstrate a direct physical interaction of the calcium binding protein CaM with ER. These two sets of results demonstrate for the first time a direct interaction of ER and CaM in a purified system.

**CaM Depletion from Nuclear Extract of MDA-MB-231 Cells Prevents Reconstitution of the ER-ERE Complex**—The above results demonstrate that CaM and ER are integral components of the ER-ERE complex. To examine a requirement for CaM in the formation of the initial ER-CaM complex, we depleted CaM from nuclear extract of MDA-MB-231 cells. Results of Fig. 6A demonstrate that this extract plus pure ER was incapable of reconstituting the ER-ERE complex (lane 3), which was generated with equivalent amounts of undepleted nuclear extract (lane 2). Addition of exogenous CaM did not restore the complex formation (Fig. 6B, lane 4), suggesting that although CaM is essential, it alone is not sufficient, and other necessary nuclear proteins are removed by co-precipitation with antibody. In this context, calmodulin binding protein CaM-BP68, which is involved in CaM action (31, 32), together with CaM and ER also failed to generate any ER-ERE complex in the reconstituted system (data not shown).

We used the same experimental strategy with native ER or CaM individually depleted from the nuclear extract of ER-MCF-7 cells (Fig. 6B). ER-ERE complex formation was significantly reduced in comparison with undepleted extract (Fig. 6B, lane 2) when ER-depleted (Fig. 6B, lanes 3–6) or CaM-depleted (Fig. 6B, lanes 7–10) nuclear extracts were used. These extracts did not form the complex even in the presence of externally added pure ER (Fig. 6B, lanes 4 and 8), or CaM (Fig. 6B, lanes 5 and 9). CaM-binding protein CaM-BP68 was not able to restore this ER-ERE complex formation, as shown in Fig. 6B.

![Diagram](https://example.com/diagram.png)
lanes 6 and 10. These results demonstrate that although CaM is an integral component of the ER-ERE complex, it also has a functional role in assembly of the complex, formed either with exogenously added pure recombinant ER or endogenous ER, and its interaction with the DNA motif.

The requirement of CaM for complex formation was studied by using a CaM-specific inhibitor, W7 (33). MCF-7 cells were grown in DCC medium for 48 h and then treated with indicated concentrations of W7 in the presence of E2 (1 × 10⁻⁷ M) for 2 h. Nuclear extracts were prepared from the control and treated cells, followed by ER-ERE complex formation analysis by EMSA as described under “Experimental Procedures.” Results presented in Fig. 7A demonstrate inhibition of ER-ERE complex formation by this CaM-specific inhibitor, supporting the postulation that CaM is essential for the formation of native protein complex with ERE. Similarly, W7 blocked complex formation when added to the reaction mixtures of the reconstituted system with pure recombinant ER plus nuclear extracts from MDA-MB-231 cells (Fig. 7B).

Functional Role of CaM in ER-ERE Complex Formation and Transactivation of E2 Responsive Promoter—The role of CaM in E2-responsive promoter activity was examined by transfection of MCF-7 cells with a recombinant plasmid construct of the E2-responsive PS2 promoter fused to the bacterial CAT reporter gene. Procedures for transfection of cells with the recombinant plasmid, treatment of the transfected cells with E2 and W7, preparation of cell extracts, assay of CAT activity, and quantitation are described under “Experimental Procedures.” Results of Fig. 7C demonstrate that W7 blocked E2-mediated transactivation of the E2-responsive PS2 promoter, again suggesting a functional role of CaM in E2- and ER-mediated regulation of responsive promoter activity. These inhibitory effects of W7 on ER-CaM auxiliary protein complex interaction with ERE and transactivation of a target gene were observed at nontoxic concentrations of the compound (Fig. 7D).

DISCUSSION

Our results show that the calcium binding protein CaM is directly involved in the key event of ER binding to the cognate DNA sequence that mediates subsequent gene transactivations. We show that CaM interacts directly with ER and in association with other auxiliary proteins mediates the downstream functions of the E2-ER pathway of hormone action and regulation of target gene expression. The several experimental approaches to establish this postulation include (a) detection of a supershifted ER-CaM-ERE complex (Fig. 1), (b) blocking formation of this complex by specific inhibitors W7 and EGTA (Figs. 2 and 7), (c) immunodepletion experiments (Figs. 3 and 6), (d) co-immunoprecipitation experiments (Figs. 4 and 5), and (e) detection of an ER-CaM complex in a nondenaturing gel electrophoretic analysis (Fig. 5).

Association of CaM with ER has been proposed from biochemical experiments with a uterine partially purified ER fraction, but the involvement of other proteins in this interaction was not eliminated (11, 16–19). Although CaM has been implicated in the phosphorylation of ER (15), its role in a molecular downstream event such as ER-ERE complex formation and transactivation of a responsive gene has not been directly demonstrated.

The inability of CaM and CaM-BP68 to restore the ER-ERE complex in ER- or CaM-immunodepleted extracts demonstrates that other auxiliary proteins are required (34, 35).
These are yet to be identified. Two classes of proteins are involved in E2-mediated transactivation of hormone-responsive genes. One class, including CaM, is active in generating a protein-protein complex that interacts with the specific DNA sequence to form the ER×ERE complex. These auxiliary proteins are different from transcriptional coactivators, the second class of proteins that are involved in transactivation events downstream of formation of the ER auxiliary protein-ERE complex and are proximal to the basic transcriptional machinery (36–41). Involvement of cyclin D1 in ER-mediated transactivation has been reported by exogenously introducing cyclin D1 by transfection (34, 35). The role of endogenous cyclin D1 has not yet been clearly demonstrated.

All of the results presented demonstrate that the Ca2+-binding protein CaM plays an active role in assembly of the ER-ERE complex, and that it is fundamental to E2-regulated gene expression in breast cancer cells. Disregulation of Ca2+ homeostasis and modulation of expression of the class of genes encoding calcium binding proteins such as CaM, reticulocalbin, and Mts1 are associated with breast cancer cell proliferation and apoptosis (1–4, 6, 42–45). Synergistic inhibition of growth by CaM inhibitors and antiestrogens has been reported (12, 13). These results provide a rationale for simultaneously targeting both ER and CaM in chemotherapeutic treatment of breast cancers.

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FIG. 7. Influence of CaM-inhibitor W7 on ER-ERE interaction and PS2-promoter activation. A, ER-32P-ERE complex formation with cellular (native) ER in the nuclear extracts of control and W7-treated E2-activated MCF-7 cells grown in 100-mm tissue culture dishes in 10 ml of complete medium as described under “Experimental Procedures.” Twenty-four hours later the rich medium was removed, washed once with DCC medium, and replenished with 10 ml of fresh DCC medium. Forty-eight hours later the cells were treated with indicated concentrations of E2 alone or in the presence of W7. Two hours later the cells were washed and harvested, and nuclear extracts were made (23). ER-32P-ERE interaction with equal amounts (protein) of control and drug-treated nuclear extracts was then measured by EMSA as described in the legend to Fig. 1. The intensity of the retarded ER-32P-ERE band was quantitated by densitometric scanning and integration as described under “Experimental Procedures.” The band intensity of the control ER-32P-ERE complex in the presence of E2 and absence of the drug was represented as 100% and in the drug-treated ER-ERE complex bands as relative to the control. B, formation of ER-32P-ERE complex reconstituted with pure human recombinant ER and nuclear extracts from ER-deficient MDA-MB-231 cells in the presence of indicated concentrations of W7 as analyzed by EMSA. Cell growth and nuclear extract preparation conditions were the same as described above. Quantitation of the intensity of the retarded radioactive bands was by densitometric scanning and integration as described under “Experimental Procedures.” C, inhibition of E2-responsive PS2 promoter activity by CaM inhibitor W7 in MCF-7 cells grown in DCC medium for 48 h and transfected with 10 μg of PS2-CAT fusion plasmid with SuperFect using the protocol provided by the manufacturer (Qiagen). Forty-eight hours after transfection the cells were treated with the indicated concentrations of E2 or W7 either singly or in combination for 2 h. The cell extracts were then prepared from the control and drug-treated cells. Preparation of cell extracts and assay of CAT activity in the extracts were described previously (25). The level of CAT activity in the uninduced cells is represented as 100%, and that in E2 and W7-treated cells is presented as relative to that of the uninduced cells. CAT activity is expressed as the percentage of the acetylated [14C]chloramphenicol derivatives produced per hour in 25 μg of cell extract protein. D, viability of W7-treated cells as measured by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (46).
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