

Steroid Receptor Regulation of Epidermal Growth Factor Signaling through Src in Breast and Prostate Cancer Cells: Steroid Antagonist Action

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Abstract

Under conditions of short-term hormone deprivation, epidermal growth factor (EGF) induces DNA synthesis, cytoskeletal changes, and Src activation in MCF-7 and LNCaP cells. These effects are drastically inhibited by pure estradiol or androgen antagonists, implicating a role of the steroid receptors in these findings. Interestingly, EGF triggers rapid association of Src with androgen receptor (AR) and estradiol receptor α (ER α) in MCF-7 cells or ER β in LNCaP cells. Here, we show that, through EGF receptor (EGFR) and erb-B2, EGF induces tyrosine phosphorylation of ER preassociated with AR, thereby triggering the assembly of ER/AR with Src and EGFR. Remarkably, experiments in Cos cells show that this complex stimulates EGF-triggered EGFR tyrosine phosphorylation. In turn, estradiol and androgen antagonists, through the Src-associated receptors, prevent Src activation by EGF and heavily reduce EGFR tyrosine phosphorylation and the subsequent multiple effects, including DNA synthesis and cytoskeletal changes in MCF-7 cells. In addition, knockdown of ER α or AR gene by small interfering RNA (siRNA) almost abolishes EGFR tyrosine phosphorylation and DNA synthesis in EGF-treated MCF-7 cells. The present findings reveal that steroid receptors have a key role in EGF signaling. EGFR tyrosine phosphorylation, depending on Src, is a part of this mechanism. Understanding of EGF-triggered growth and invasiveness of mammary and prostate cancer cells expressing steroid receptors is enhanced by this report, which reveals novel aspects of steroid receptor action. (Cancer Res 2005; 65(22): 10585-93)

Introduction

Growth factors and steroid receptors control breast cancer progression. In the absence of estradiol, epidermal growth factor (EGF) activity converges on the estradiol receptor (ER) in human mammary cancer-derived cells, as well as in uterus, thereby triggering DNA synthesis and cell proliferation. EGF can also activate genes regulated by estrogen-responsive elements (1–3). In addition, growth factors directly activate androgen receptor (AR) in androgen-deprived prostate cancer cells (4). An increase in

uterine weight and proliferation of the uterine epithelial cells follows EGF or insulin-like growth factor-I (IGF-I) treatment of ovariectomized mice. Interestingly, these effects are not observed in ER α knockout mice, indicating that ER α is required in this case (3).

Tyrosine kinases are involved in human breast cancer development and EGF receptor (EGFR) is amplified in 10% to 35% of human breast carcinomas. This event is correlated with a poor disease prognosis (5). Elevated Src activity has been found in breast tumor specimens and cell lines (6). Moreover, both EGFR and Src are overexpressed in a subset of human breast tumors and a wealth of evidence indicates physical and functional associations between EGFR and Src (reviewed in ref. 7). Expression of a dominant-negative form of Src in murine fibroblasts interferes with EGF-induced mitogenesis and cytoskeletal changes (8). In addition, Src has been implicated in the regulation of EGFR endocytosis (9). Therefore, Src seems to be a component of the signaling pathway elicited by EGF.

MCF-7 and LNCaP cells are hormone-responsive cells derived from human mammary and prostate cancers, respectively. They express AR and either ER α (MCF-7) or ER β (LNCaP) and are widely used to analyze the effect of sex-steroid hormones and the cross-talk between growth factors and steroid hormones. We previously reported that in MCF-7 and LNCaP cells, ER and AR, once activated by steroid hormones, stimulate a mitogenic signaling network known to be engaged by growth factors (10, 11). In this report, we attempted to shed light on the cross-talk between EGFR and steroid receptors and to determine the effect of the steroid antagonists on mammary and prostate cancer cells in the absence of steroid hormones. We now observe that in the two human cancer-derived cell lines, under conditions of short-term hormone deprivation, specific steroid antagonists suppress EGF-induced DNA synthesis, cytoskeletal changes, and Src activation. Our analysis of the effect of these antagonists revealed unexpected features in the cross-talk between EGFR and steroid receptors. EGF induced ER/AR/Src complex in MCF-7 and LNCaP cells. A small percentage of the total AR, ER α , or ER β was found to be associated in these cells under basal conditions. These preassociated receptors, together with ER tyrosine phosphorylation elicited by EGF, play a major role in the heterodimer steroid receptor interaction with Src and the regulation of Src-dependent action. We suggest that once Src-associated receptors are occupied by steroid antagonists, they interfere with the EGF-induced active AR/ER/Src complex, thereby silencing their effects on Src. One of these, and probably the most unexpected, is EGFR tyrosine phosphorylation. Remarkably, ER α and AR knockdown by small interfering RNA (siRNA) heavily reduces EGFR tyrosine phosphorylation as well as DNA synthesis stimulated by EGF in MCF-7 cells.

Note: A. Migliaccio and M. Di Domenico contributed equally to this work.

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The role of the steroid receptors in EGF signaling is further stressed by experiments in Cos cells showing that EGFR tyrosine phosphorylation triggered by EGF is strongly up-regulated by transient expression of ER α and AR, provided that the AR/ER/Src complex can be assembled.

Materials and Methods

Constructs and small interfering RNA. cDNAs encoding hAR, wild-type hER (HEGO), HEG241, and the point-mutated (HEG537F) forms of hER α were cloned into pSG5 expression vector as described previously (11–14). The wild-type and the kinase-inactive form of Src (Lys²⁵⁹ changed to methionine) were cloned into pSG5 as reported (15). Smart pools of double-stranded siRNA against AR as well as nonspecific siRNAs were obtained from Dharmacon Tech (Lafayette, CO). The stealth RNA interference (RNAi) against ER α and the stealth RNAi-negative control were from Invitrogen (Carlsbad, CA). The negative control siRNA Alexa Fluor 488 was from Qiagen (Valencia, CA). Specific and nonspecific siRNA were used according to the instructions of the manufacturer.

Cell culture and transfection techniques. Human mammary cancer-derived MCF-7 and MDA-MB231 cells were grown and made quiescent as reported elsewhere (16). Human prostate cancer-derived LNCaP and Cos cells were grown and made quiescent as previously reported (10, 11). When indicated, Cos cells were made quiescent by serum starvation (17). Quiescent cells were transfected by Superfect (Qiagen) using 2 μ g of purified plasmids. Twenty-four hours later, transfected cells were left unstimulated or stimulated with the indicated compounds. For the siRNA studies, MCF-7 cells at 70% confluence were transfected in complete medium with LipofectAMINE 2000 Reagent (Invitrogen) according to the instructions of the manufacturer. RNAi against ER α was transfected for 48 hours, and siRNA against AR was transfected for 24 hours. Cells were then made quiescent and left unstimulated or stimulated with the indicated compounds.

DNA synthesis analysis and cytoskeletal changes. For BrdUrd incorporation analysis, quiescent cells on coverslips were left unstimulated or stimulated for 24 hours with the indicated compounds. After a 6-hour pulse with 100 μ mol/L BrdUrd (Sigma, St. Louis, MO), BrdUrd incorporation was analyzed as described (16) and nuclei were stained with Hoechst 33258 (Sigma). For the siRNA studies, the cells were cotransfected with negative control siRNA Alexa Fluor 488 (at 15 nmol/L) to help identify transfected cells and BrdUrd incorporation was analyzed as reported (16) using Alexa Fluor 594-conjugated mouse monoclonal anti-BrdUrd antibody (Molecular Probes, Eugene, OR). For cytoskeletal changes, cells on coverslips were made quiescent by serum starvation (0.1% serum) for 16 hours. They were then left unstimulated or stimulated for 20 minutes with the indicated compounds. F-actin was visualized using either Texas red or FITC-labeled phalloidin as reported (17). Finally, coverslips were mounted in Mowiol (Calbiochem, Darmstadt, Germany). Images were generated with a DMLB fluorescent microscope (Leica, Wetzlar, Germany) equipped with $\times 40$ and $\times 100$ lens and processed with IM1000 software (Leica).

Immunoprecipitation, kinase, and Ras assays. Lysates were prepared as described previously (10) and protein concentration was measured with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of cell lysates (2 mg protein/mL) were used for immunoprecipitation and kinase assay of Src and extracellular signal-regulated kinase-2 (Erk-2; ref. 10). ER α was immunoprecipitated using the anti-ER rabbit polyclonal antibody (HC-20, Santa Cruz, Santa Cruz, CA) and AR was immunoprecipitated using the anti-AR rabbit polyclonal antibody (N-20, Santa Cruz). EGFR was immunoprecipitated using the anti-EGFR rabbit polyclonal antibodies (UBI, Charlottesville, VA). The Ras activation assay kit (UBI) was used for Raf-RBD pullout.

Protein-protein interaction assay. Recombinant proteins were produced as previously described (11). Coupled *in vitro* transcription/translation reactions were used to produce ³⁵S-labeled proteins in rabbit reticulocyte lysates (Promega, Madison, VA) and protein-protein interaction assays were done according to the same report (11). Eluted proteins were resolved by SDS-PAGE and protein bands were revealed by fluorography.

Electrophoresis and immunoblotting. The electrophoresis and immunoblotting procedures were done as described elsewhere (10). Src was revealed using the mouse monoclonal anti-Src antibody (clone 327; Calbiochem), EGFR was visualized using anti-EGF receptor rabbit polyclonal antibody (UBI), and Ras was detected using anti-pan Ras antibody (UBI). P-tyrosine proteins were detected using the mouse monoclonal antiphosphotyrosine antibody (clone 2G4; UBI). The rabbit polyclonal anti-AR antibodies (either C-19 or N-20, Santa Cruz) were used to reveal AR. ER α was immunoblotted using the rabbit polyclonal anti-ER (HC-20, Santa Cruz) antibody. Modulator of nongenomic activity of estrogen receptor (MNAR) was detected using rabbit polyclonal anti-PELP1/MNAR antibody (Novus Biologicals, Littleton, CO). Immunoreactive proteins were revealed with the ECL detection system (Amersham Biosciences, Bucks, United Kingdom).

Results

Epidermal growth factor-stimulated DNA synthesis and cytoskeletal changes in MCF-7 cells are regulated by steroid receptors and Src. EGF (100 ng/mL) stimulated the S-phase entry of MCF-7 cells maintained in phenol red-free medium supplemented with charcoal-treated serum (Fig. 1A). This response was similar in intensity to that of the same cells treated with 10 nmol/L estradiol (Fig. 1A). In accordance with a previous report (1), the effect of EGF was abolished by 10 μ mol/L ICI 182,780, a pure antiestrogen. The same antagonist showed a similar effect at a 100-fold lower concentration (not shown). Because estradiol and androgen stimulate cross-talk between ER α and AR in MCF-7 cells (11), we verified the effect of a pure antiandrogen, Casodex, on the effect of EGF. Interestingly, Casodex also (10 μ mol/L) abolished the growth hormone effect (Fig. 1A). This antagonist also inhibited the G₁-S transition at a concentration of 0.1 μ mol/L (not shown). Effects similar to those of the two steroid antagonists were observed in the presence of the Src kinase family inhibitor, PP2 (Fig. 1A), as well as in cells transiently transfected with siRNA silencing ER α or AR (Fig. 1B and C).

Altogether, these findings indicate that steroid receptors and Src play a key role in EGF-triggered DNA synthesis.

EGF evokes dramatic morphologic changes and stress fiber breakdown in a number of cell types (18). Here, we show that EGF at a concentration of 100 ng/mL rapidly induces fan-like membrane protrusions and ruffles in MCF-7 cells. Also, in this case, both steroid antagonists prevented EGF-induced cytoskeletal changes (Fig. 1D). Src activity is also involved in these responses as indicated by the PP2 inhibitory effect on EGF-induced cytoskeletal changes (Fig. 1D). Findings in Fig. 1 show that the two steroid receptors and Src have a key role in the EGF-elicited effects in MCF-7 cells.

Epidermal growth factor-triggered Src activation in MCF-7 cells is inhibited by steroid antagonists. The effect of EGF on DNA synthesis and membrane ruffling is inhibited by steroid antagonists and Src inhibitor, PP2. Src family tyrosine kinases are involved in signaling of different growth factor receptors including EGFR. They can promote initiation of signaling pathways required for DNA synthesis and actin cytoskeleton rearrangements (19). Therefore, we evaluated the effect of 10 μ mol/L ICI 182,780 on EGF-stimulated Src activity. The activity was measured as enolase phosphorylation by the immunoprecipitated Src. We used control antibodies to verify that Src immunoprecipitation was specific and that the samples contained equal amounts of Src (Fig. 2A). EGF activated Src, whereas ICI 182,780 prevented this activation. Because Src acts upstream of Ras and the Ras-dependent kinase cascade, we evaluated the effect of ICI 182,780 on EGF-induced

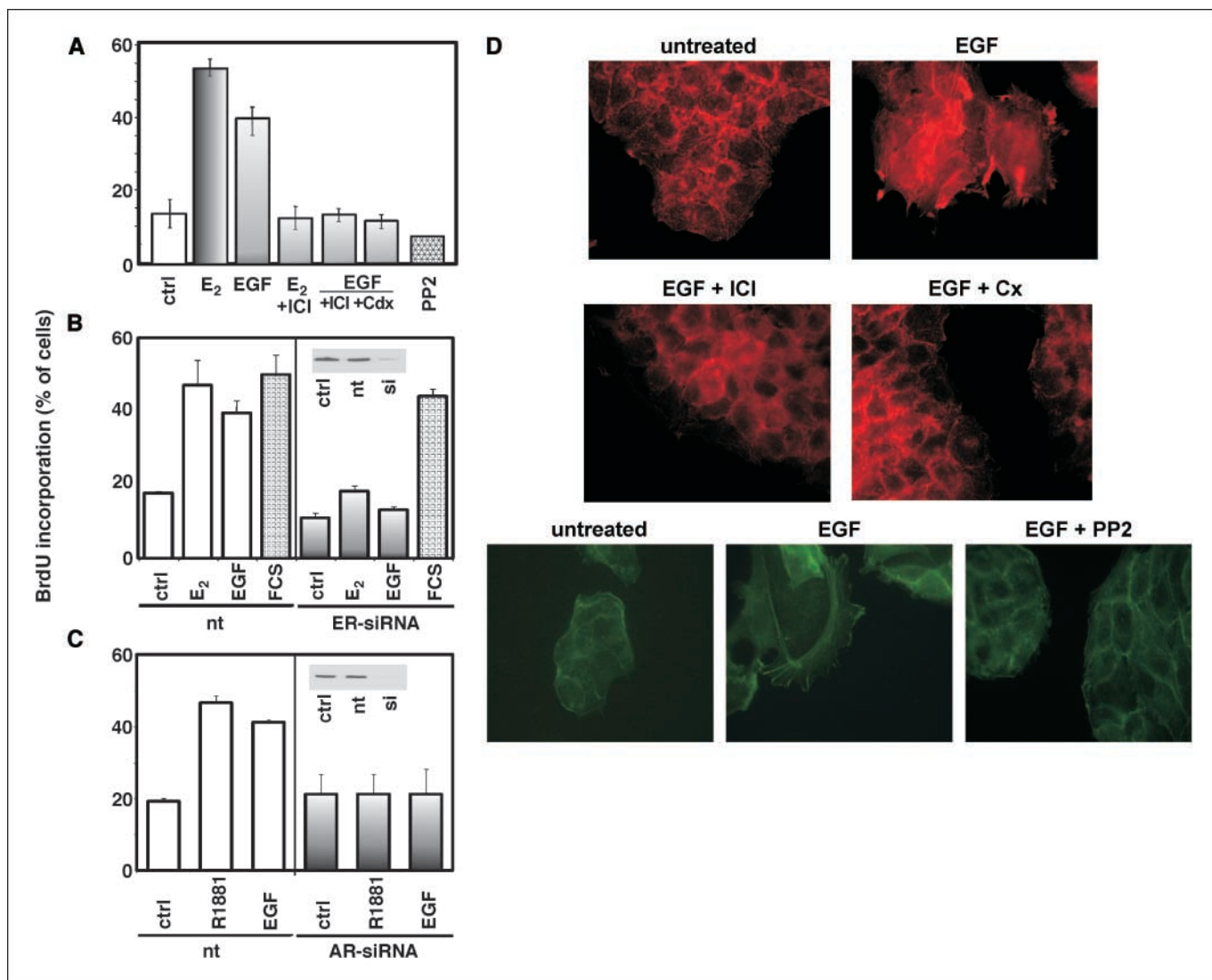


Figure 1. Steroid antagonists inhibit EGF-induced DNA synthesis and cytoskeletal changes in MCF-7 cells. **A**, quiescent MCF-7 cells were untreated or treated for 24 hours with the indicated compounds. EGF at a concentration of 100 ng/mL, estradiol (E_2) at 10 nmol/L, the antiestrogen ICI 182,780 (ICI) and the antiandrogen Casodex (Cdx) at 10 μ mol/L, and PP2 (Calbiochem) at 5 μ mol/L were added to the cell medium. After *in vivo* labeling with BrdUrd, DNA synthesis was analyzed and BrdUrd incorporation was expressed as a percentage of total nuclei. Columns, means; bars, SE. **B** and **C**, MCF-7 cells were transfected with negative control siRNA Alexa Fluor 488 together with the indicated nonspecific or specific siRNA. Cells were made quiescent by steroid depletion and then left unstimulated or stimulated for 24 hours with the indicated compounds. To assess the viability of transfected cells, MCF-7 cells were also made quiescent by serum starvation (0.1% serum) for 16 hours, then stimulated with 20% serum (FCS). After *in vivo* labeling with BrdUrd, DNA synthesis was analyzed. In transfected cells, BrdUrd incorporation was calculated by the following formula: percentage of BrdUrd-positive cells = (number of transfected BrdUrd-positive cells / number of transfected cells) \times 100. Results were compared with BrdUrd incorporation of untransfected cells from the same coverslips. For each siRNA, data are derived from at least 300 scored cells. The results of more than two independent experiments have been averaged; Columns, means; bars, SE. The insets in (**B** and **C**) show immunoblots of cell lysates with appropriate antibodies to detect the protein expression of ER α and AR. **D**, quiescent MCF-7 cells were left unstimulated or stimulated for 20 minutes with the indicated compounds. PP2 (at 5 μ mol/L) was added 30 minutes before EGF stimulation. Cells stained with either Texas red (*top and middle panels*) or FITC-conjugated (*bottom panels*) phalloidin were visualized by a fluorescent microscope using a \times 100 lens. Micrographs are representative of three independent experiments.

Ras and Erk-2 activation. The estradiol antagonist reduced the robust activation by EGF of Ras and Erk-2 (Fig. 2A). These findings indicate that ER α plays a major role in the regulation of EGF-elicited signaling. In addition to preventing Src activation by the synthetic androgen, R1881, Casodex also prevented the EGF-induced activation of Src in MCF-7 cells, which express AR (Fig. 2B).

We then used AR-positive and ER-negative MDA-MB231 cells to verify whether ER is directly involved in EGF-stimulated signaling, as suggested by the inhibitory effect of antiestrogen. Although EGF was a stimulus of Src activity in these cells, Src activation

was unaffected by ICI 182,780 but was abolished by Casodex (Fig. 2C, left). This effect of Casodex confirms the crucial role of steroid receptors on Src activity during EGF signaling. In contrast, the antiestrogen completely inhibited Src activity in MDA-MB231 cells forced to express ER α (Fig. 2C, right). Therefore, we conclude that inhibition of EGF-induced Src activation by ICI 182,780 requires ER α expression.

Epidermal growth factor induces rapid estradiol receptor α and androgen receptor association with Src and epidermal growth factor receptor in MCF-7 cells and in transiently transfected Cos cells. Estradiol and androgen induce rapid

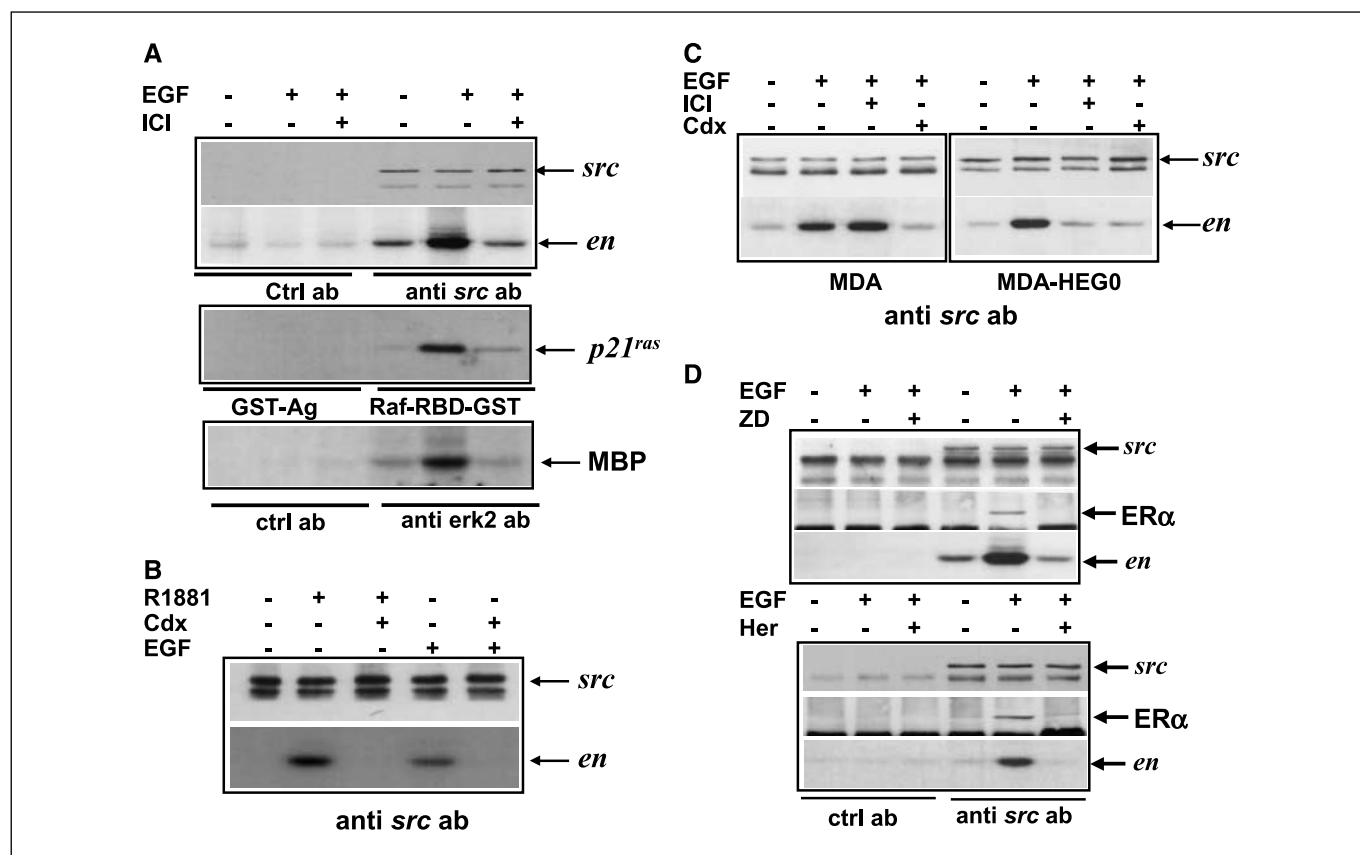


Figure 2. Inhibition by steroid antagonists and Iressa (ZD 1839) or herceptin (Trastuzumab) of EGF-induced Src activation in MCF-7 cells. ER α requirement for the antiestrogen effect. **A** and **B**, quiescent MCF-7 cells were left unstimulated or stimulated for 5 minutes with the indicated compounds. The antiestrogen ICI 182,780 and the antiandrogen Casodex were used at a concentration of 10 μ mol/L, EGF at 100 ng/mL, and R1881 at 10 nmol/L. Lysate proteins were immunoprecipitated with either anti-Src (*anti-src ab*) or control (*Ctrl ab*) antibodies. The immunoprecipitates were either blotted with anti-Src antibody (*top panels in A and B*) or analyzed for Src activity using enolase (*en*) as a substrate (*top panels in A and B*). **A**, *middle*, Ras pull-down assay of MCF-7 cell lysates. **A**, *bottom*, Erk-2 activity assay done using myelin basic protein (*MBP*) as a substrate. **C**, quiescent MDA-MB231 cells were left unstimulated or stimulated for 5 minutes with the indicated compounds. Src activity was assayed in untransfected (*MDA*) or hER α -transfected (*MDA-HEG0*) cells. **D**, MCF-7 cells, pretreated with 0.5 μ mol/L Iressa (*ZD*) or 10 μ g herceptin (*Her*), were stimulated with EGF for 5 minutes. Lysate proteins were immunoprecipitated with either control or anti-Src antibodies. Immunocomplexes were either blotted with antibodies against the indicated proteins (Src or ER α) or submitted to Src kinase assay.

association of Src with AR and ER β or ER α in LNCaP and MCF-7 cells, respectively (11). Phosphotyrosine 537 of ER α directly interacts with the Src-SH2 domain and is required for ER/Src association (11). Here, we show that, simultaneous to Src activation, EGF induces or increases the association of ER α and AR with Src in MCF-7 cells (Fig. 2D and 3C, respectively).

We next investigated the role of EGFR-tyrosine kinase and erb-B2 in Src/ER α /AR association. A selective inhibitor of EGFR-tyrosine kinase, Iressa (ZD 1839), blocked EGF-elicited Src activity (Fig. 2D). It also prevented the association of Src with ER and AR (Fig. 2D and 3C, respectively). Preincubation of MCF-7 cells with the anti-erb-B2 antibody herceptin (Trastuzumab) produced the same effects [i.e., inhibition of Src activity (Fig. 2D) and reduction of association of Src with ER α and AR (Fig. 2D and 3C, respectively)]. The growth factor induced tyrosine phosphorylation of ER α (Fig. 3A and B).

The parallel inhibition of the steroid receptor/Src complex and ER tyrosine phosphorylation by Iressa (Fig. 3A) and herceptin (Fig. 3B) suggests that these two events are causally related. To address this point, we transiently transfected Cos cells with hAR and hER α (HEG0) cDNAs. The HEG537 cDNA mutant, lacking the only phosphorylatable tyrosine (20), was also expressed instead

of wild-type ER α (Fig. 3D, *left*). Cells were then stimulated with EGF and lysates were immunoprecipitated with anti-Src antibody. Immunoblotting revealed that the samples contained equal amounts of Src (Fig. 3D, *right, top*). As shown in Fig. 3D (*right*), EGF was a potent inducer of the Src/AR/ER α complex in cells expressing wild-type ER α . The association with Src of the mutant was very weak and that of AR was absent, which shows that ER α tyrosine phosphorylation in position 537 is required for the coexpressed receptors/Src complex assembly.

When MCF-7 cells were stimulated by EGF for 5 minutes, association of the EGFR with ER, AR, Src, and MNAR was detectable. It has been reported that MNAR modulates the estradiol-dependent ER interaction with Src (21). Its association in the EGFR/ER/AR/Src complex triggered by EGF supports the view that this protein interacts with activated ER. In the absence of the growth factor, no association of the EGFR with these proteins was observed (Fig. 4A, *left*).

In addition, in Cos cells transiently expressing hER α or its mutant HE241G, EGF induces association of its own receptor not only with the wild-type hER α but also with the HEG241 mutant (Fig. 4A, *right*). Because the HE241G mutant does not bind DNA, lacks the nuclear localization signal (14), and permanently resides

outside the nucleus of MCF-7 cells,¹ ER α associated with EGFR must be in the extranuclear compartment of cells. This finding is in agreement with the involvement in the EGF-triggered EGFR/ER/AR/Src complex of Src, which is a classic cytoplasmic protein, and the role of steroid receptors in the EGF-triggered cytoskeletal changes, which are expressed independently of the cell nucleus (22).

Androgen receptor and estradiol receptor α are associated in MCF-7 cells. An association between AR and ER α but not Src was observed in unstimulated MCF-7 cells (not shown). To evaluate the proportion of AR and ER α in the complex, we coimmunoprecipitated the receptors from MCF cell lysates with anti-ER α or anti-AR antibodies. Supernatants were collected for further analysis, and the immunocomplexes were eluted. Lysates, breakthroughs, and immunocomplexes were probed by Western blots with anti-AR or anti-ER α antibodies (Fig. 4B). The band intensities were measured and the percentage of associated proteins was calculated as described in Fig. 4 caption. About 8% of the total AR and ER α were associated. Interestingly, similar results were observed with LNCaP cells using immunoprecipitating anti-ER β instead of anti-ER α antibodies (not shown).

We then conducted pulldown experiments with glutathione *S*-transferase (GST) fusion protein constructs to determine whether the association between the two receptors was direct (Fig. 4C). GST-agarose, GST-HEG0 (HEG0 is the entire hER α), GST-HEG14 (HEG14 is the carboxyl-terminal half of hER α containing the hormone binding domain), and GST-HEG15 (HEG15 is the amino-terminal half of hER α) were incubated with the [³⁵S]methionine-labeled hAR. Association of AR with GST-HEG14 was comparable with the low, nonspecific interaction of AR with GST-agarose, whereas there was a much stronger association with the entire receptor (GST-HEG0) and the amino-terminal half of ER α , GST-HEG15. These experiments reveal a direct interaction between the ER α amino-terminal half and AR in unstimulated cells.

Epidermal growth factor-stimulated epidermal growth factor receptor tyrosine phosphorylation in MCF-7 cells is regulated by steroid receptors and Src. The foregoing experiments show that EGFR regulates ligand-independent steroid receptors and Src activity. We conducted experiments to determine whether steroid receptors in turn regulate the EGFR. To this end, quiescent MCF-7 cells were stimulated or not with EGF alone or in the presence of 10 μ mol/L ICI 182,780 or 10 μ mol/L Casodex. Cell lysates were then immunoprecipitated with anti-EGFR antibody and the immunocomplexes probed with the same antibody or with antiphosphotyrosine antibodies (Fig. 5A). As expected, Western blot analysis showed that EGF stimulated phosphorylation of its own receptor. Remarkably, EGFR phosphorylation was strongly reduced when the cells were stimulated by the growth factor in the presence of antihormones. This finding suggests a novel, steroid-independent regulatory role of steroid receptors on EGFR. This conclusion is further confirmed by the strong inhibitory effect on EGFR phosphorylation detected after the knockdown of ER α or AR gene in MCF-7 cells (Fig. 5B).

We then conducted experiments to verify whether Src was involved in EGFR phosphorylation in MCF-7 cells. In the experiment shown in Fig. 5C, cells were transfected with the wild-type Src or with a kinase-inactive Src and then stimulated with EGF. The growth factor induced rapid EGFR tyrosine phosphorylation in cells expressing wild-type Src. This phosphorylation was much

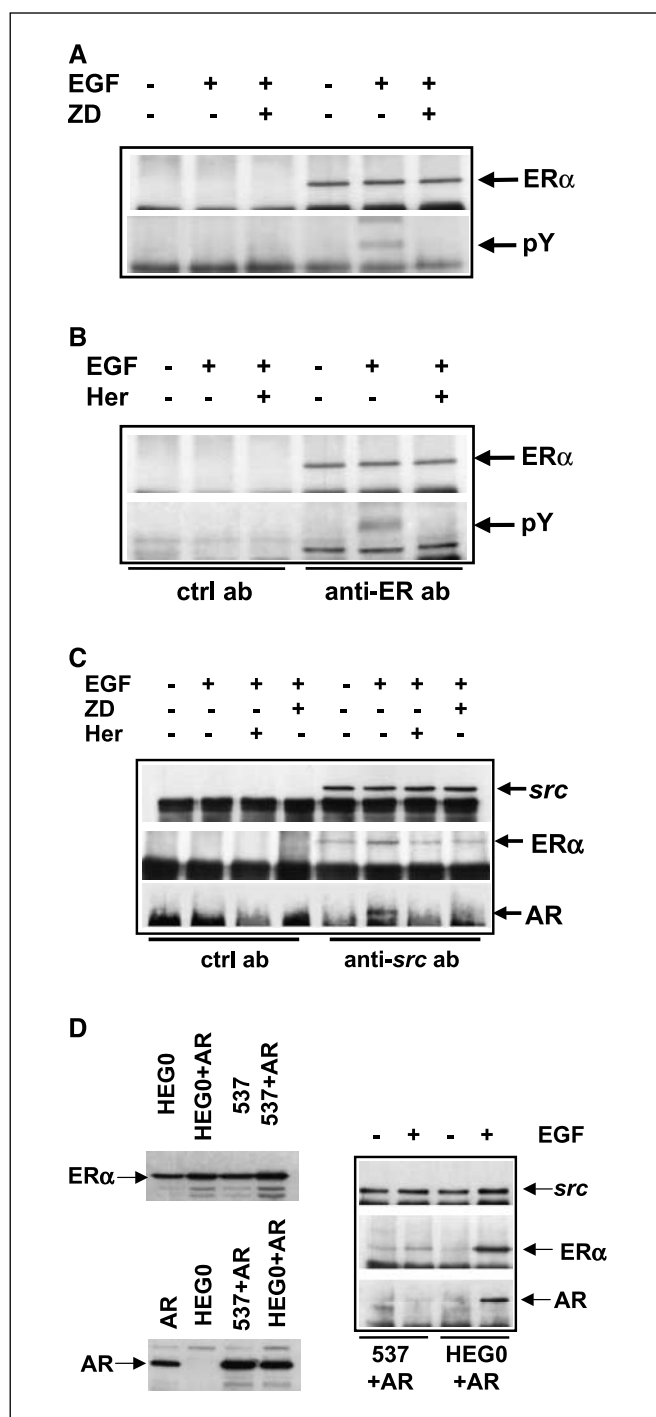


Figure 3. Role of phosphorylation on ER α tyrosine 537 in the EGF-elicited ER/AR/Src association in MCF-7 and Cos cells. *A* to *D*, quiescent MCF-7 cells were untreated or treated with EGF (100 ng/mL) for 5 minutes in the absence or presence of the indicated compounds. Iressa and herceptin were used at 0.5 μ mol/L and 10 μ g/mL, respectively. The antiestrogen was used at 10 μ mol/L. *A* and *B*, lysates were immunoprecipitated with either anti-ER (*anti-ER ab*) or control antibodies. *C*, lysates were immunoprecipitated with either anti-Src or control antibodies. Immunoprecipitated proteins were blotted with the antibodies against the indicated proteins or antiphosphotyrosine (*pY*) antibodies. *D*, Cos cells were transfected with the indicated plasmids and then left unstimulated or stimulated for 5 minutes with 100 ng/mL EGF. Lysates were immunoprecipitated with anti-Src antibodies and immunocomplexes were blotted with antibodies against the indicated proteins (*right*). *Left*, expression of steroid receptors evaluated by immunoblots with appropriate antibodies. HEG0 is the hER α , 537 is the hER α carrying the Y537F mutation, and AR is the hAR.

¹ In preparation.

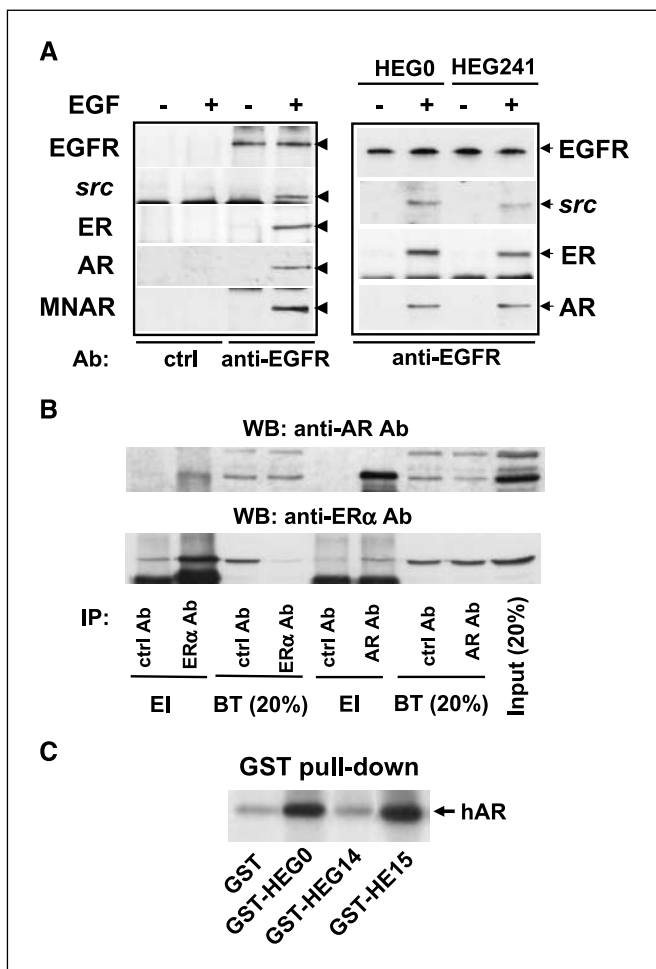


Figure 4. Protein association in MCF-7 cells and *in vitro*. *A, left*, quiescent MCF-7 cells were untreated or treated with EGF (100 ng/mL) for 5 minutes. Lysates were incubated with control or anti-EGFR antibody (Ab). The immunoprecipitates were blotted with antibodies raised against the indicated proteins. *Right*, Cos cells were transfected with wild-type hER α (HEGO) or its mutant (HEG241) and were then stimulated with 100 ng EGF for 5 minutes. Lysates were immunoprecipitated and blotted as in *A, left*. *B*, unstimulated MCF-7 cells were used. Lysates were immunoprecipitated with either anti-ER α or anti-AR antibodies. Loaded samples (*Input*), breakthroughs (*BT*), and immunocomplexes (*EI*) were blotted with antibodies against the indicated proteins. A NIH 1.61 image program showed that 8% of the total AR and ER were associated under basal conditions. *C*, GST-agarose (GST), GST-HEGO, GST-HEG14, and GST-HEG15 were incubated with ^{35}S -labeled AR. Eluted proteins were subjected to SDS-PAGE and revealed by fluorography. HEG15 and HEG14 are the amino-terminal half and the carboxyl-terminal half of HEGO (hER α), respectively.

weaker in cells expressing kinase-inactive Src. All together, these results are consistent with the view that Src kinase activity plays a key role in the EGF-dependent EGFR phosphorylation in MCF-7 cells and suggest that this activity is under the control of the Src-associated steroid receptors. This possibility is strongly corroborated by the experiments in Fig. 5D. Cos cells were transiently cotransfected with hAR (AR) and the wild-type hER α (HEGO) or its mutant HEG537 (537), which lacks the property of interacting with Src in Cos cells stimulated by EGF (see Fig. 3D). EGF strongly stimulated EGFR tyrosine phosphorylation in Cos cells expressing HEGO, whereas a much weaker stimulation was detected in cells expressing HEG537. This shows that the ER/AR/Src complex has a key role in EGFR tyrosine phosphorylation. Remarkably, comparison of EGFR tyrosine phosphorylation in cells transfected with

the empty vector or ER α - and AR-expressing plasmids shows that in the presence of the two steroid receptors a much stronger EGFR phosphorylation is triggered by EGF (Fig. 5D).

Epidermal growth factor-elicited effects are inhibited by steroid antagonists in LNCaP cells. In LNCaP cells, like in MCF-7 cells, EGF induced DNA synthesis. This response was similar to that evoked in the same cells by the synthetic androgen R1881. Antiandrogen and antiestrogen abolished this stimulation (Fig. 6A). The antagonists also prevented EGF-induced cytoskeleton changes (Fig. 6B) and the growth factor-stimulated Src activation (Fig. 6C). EGF induced association of ER β and AR with Src. This association was inhibited by steroid antagonists (Fig. 6C). These findings slightly indicate that mammary cancer cells and prostate cancer cells are similarly affected by EGF and steroid antagonists.

Discussion

Steroid receptors can be activated in the absence of their cognate ligands. Dopamine indeed causes nuclear translocation of the chicken progesterone receptor (23). EGF enhances nuclear localization of ER α , induces a nuclear form of ER α , and stimulates DNA synthesis in mouse uterus (2). Remarkably, such a stimulation requires the expression of ER α (3). Heregulin induces tyrosine phosphorylation of ER α in MCF-7 cells, interaction of ER α with estrogen responsive element, and progesterone receptor induction (24). EGF stimulates serine and tyrosine phosphorylation of ER α and its coimmunoprecipitation with EGFR (25). Androgen receptor is transcriptionally activated in a ligand-independent manner by IGF-I, EGF, and keratinocyte growth factor in human prostate tumor cell lines (26). HER-2/neu activates the AR pathway in the absence of ligand and increases AR responses in the presence of low levels of androgen in prostate cancer cells (4). From these and many other reports, it seems that cross-talk between growth factors and steroid receptors, which occurs at multiple levels, is involved in cancer progression and in endocrine resistance (27, 28).

Here, we report evidence of a novel interplay between the EGF-activated EGFR, its partner erb-B2, and steroidal receptors. In this regard, it is noteworthy that an EGFR/erb-B2 heterocomplex has been identified (29) and an association between erb-B2 and Src has been detected in human breast cancer cell lines (see references in ref. 7). The results concerning EGFR tyrosine phosphorylation warrant particular attention. In MCF-7 cells, the EGF-stimulated phosphorylation of EGFR was greatly reduced by either ER and AR antagonists or knockdown by siRNA of each of the two steroid receptors. The role of the two steroid receptors in this phosphorylation is further underlined by the observation that in Cos cells, EGFR tyrosine phosphorylation is greatly increased by expression of ER α and AR.

Present findings suggest that in MCF-7 and LNCaP cells steroid receptors regulate EGFR receptor phosphorylation through Src. Such a hypothesis is supported by the EGF-triggered association of EGFR with ER α , AR, and Src. In addition, this phosphorylation is almost abolished in Cos cells by expression of the ER α mutant HEG537, which lacks the ability of interaction with Src. Altogether, the present findings indicate that the ER α /AR/Src association is responsible for the Src-dependent EGFR tyrosine phosphorylation. As a part of the described regulatory mechanism, EGF treatment of hormone-responsive cells led to ER tyrosine phosphorylation and triggered strong association of Src with AR and ER α or ER β in MCF-7 and LNCaP cells, respectively. The crucial role of Src in EGF signaling is supported by the inhibition of EGF-triggered DNA synthesis and cytoskeletal changes observed in MCF-7 cells treated with the Src kinase family inhibitor, PP2, and the great reduction

of EGFR tyrosine phosphorylation in MCF-7 cells transfected with a dominant-negative form of Src.

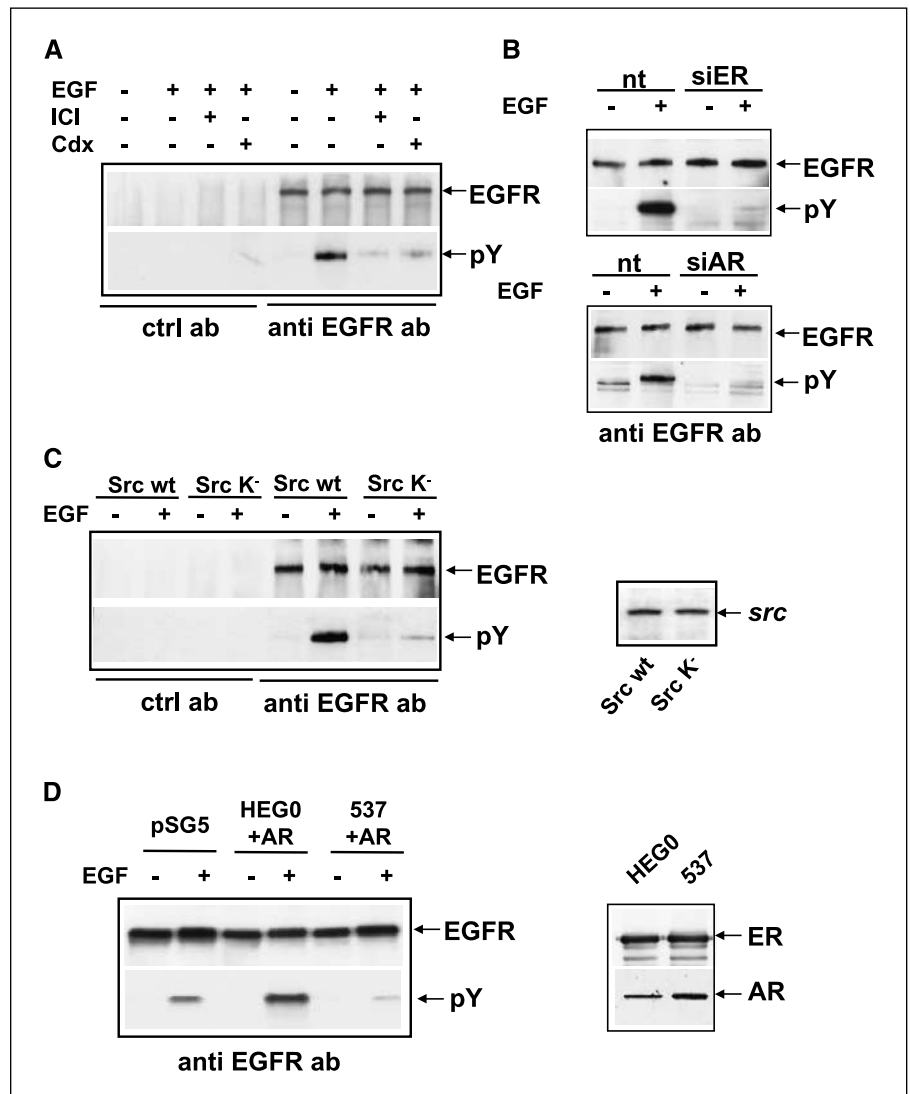
Based on these results, one may envisage a new model of cross-talk between steroid receptors and EGFR with a central role of the physical and functional interactions between EGFR, steroidal receptors, and Src. The EGF-activated Src, which is associated with ER/AR dimer, acts strongly on EGFR phosphorylation. Conversely, when ER and/or AR are locked in an inactive conformation (i.e., by hormone antagonists or when the steroid receptor levels are down-regulated by siRNA), their action on Src and EGFR is missing or heavily impaired and EGF-induced EGFR tyrosine phosphorylation is minimal. Interestingly, in MCF-7 cells, steroid antagonists and silencing of steroid receptor genes abolished the EGF-elicited DNA synthesis, thus indicating that such an effect requires steroid receptors. Similarly, ER α is required for EGF-triggered DNA synthesis in uterine epithelial cells (3).

Our report indicates that steroidal receptors play a dominant role in regulating the functions of hormone-dependent cells, even in the absence of steroids, and that ER and AR represent checkpoints of EGF signaling.

The present findings call for additional comments. The complexity of the described cross-talk between EGF and the steroid receptor/

Src complex is underlined by the observation that steroid receptors also control, through Src, the EGF-elicited cytoskeleton changes, a nongenomic effect in breast and prostate cancer cells. Association of AR with ER in MCF-7 and LNCaP cells under basal conditions represents another novel and, in our opinion, important aspect of the cross-talk between the two receptors previously analyzed (11). This study also reveals other aspects of the molecular assembly that govern nongenomic steroid receptor action. In the ER/AR/Src complex triggered by estradiol or androgen in MCF7, LNCaP, and T47D cells (11), phosphotyrosine in position 537 of ER α is crucial for the hormone-induced association of ER α with Src-SH2 and consequent Src activation and mitogenesis (11). The same phosphotyrosine residue is required for the association of ER α with Src triggered by EGF. Indeed, treatment of MCF-7 cells with Iressa or herceptin blocked EGF-stimulated tyrosine phosphorylation of ER α and the ER α /AR association with Src. It is worth mentioning, in this regard, that ER α in MCF-7 cells has a single phosphotyrosine residue in position 537 (30). Furthermore, the failure of EGF to induce the ER/AR/Src association in Cos cells cotransfected with AR and HEG537 cDNA shows that the ER phosphotyrosine in position 537 is the residue involved in the EGF-induced receptor association with Src.

Figure 5. EGFR tyrosine phosphorylation triggered by EGF is regulated by the ER/AR/Src complex. **A**, quiescent MCF-7 cells were untreated or treated for 5 minutes with the indicated compounds. EGF at a concentration of 100 ng/mL, the antiestrogen ICI 182,780, and the antiandrogen Casodex at a concentration of 10 μ mol/L were added to the cell medium. Cell lysates were immunoprecipitated with control or anti-EGFR antibodies (*anti-EGFR ab*). Immunoprecipitates were either blotted with anti-EGFR antibodies or monoclonal antiphosphotyrosine antibodies. **B**, MCF-7 cells were transfected with control, nontargeting siRNA (*nt*), and ER targeting siRNA (*siER*) or AR-targeting siRNA (*siAR*), then stimulated with 100 ng EGF for 5 minutes. Cell lysates were immunoprecipitated and blotted like in (A). **C**, MCF-7 cells were transfected with the indicated plasmids and were then left unstimulated or stimulated with 100 ng/mL EGF. Lysates were either analyzed for the expression of wild-type Src and kinase-inactive Src by immunoblots with the appropriate antibody (*right*) or immunoprecipitated with control or anti-EGFR antibodies (*left*). The immunocomplexes were then blotted with anti-EGFR or antiphosphotyrosine antibodies (*left*). **D**, Cos cells were transfected with the indicated plasmids and then made quiescent by serum starvation. The cells were left unstimulated or stimulated for 5 minutes with 100 ng/mL EGF. Lysates were immunoprecipitated with anti-EGFR antibody. The immunocomplexes were then blotted with anti-EGFR or antiphosphotyrosine antibodies (*left*). Coexpression of steroid receptors (wild-type hER α or AR or hER α -537 mutant) was verified by immunoblots with the appropriate antibodies (*right*).



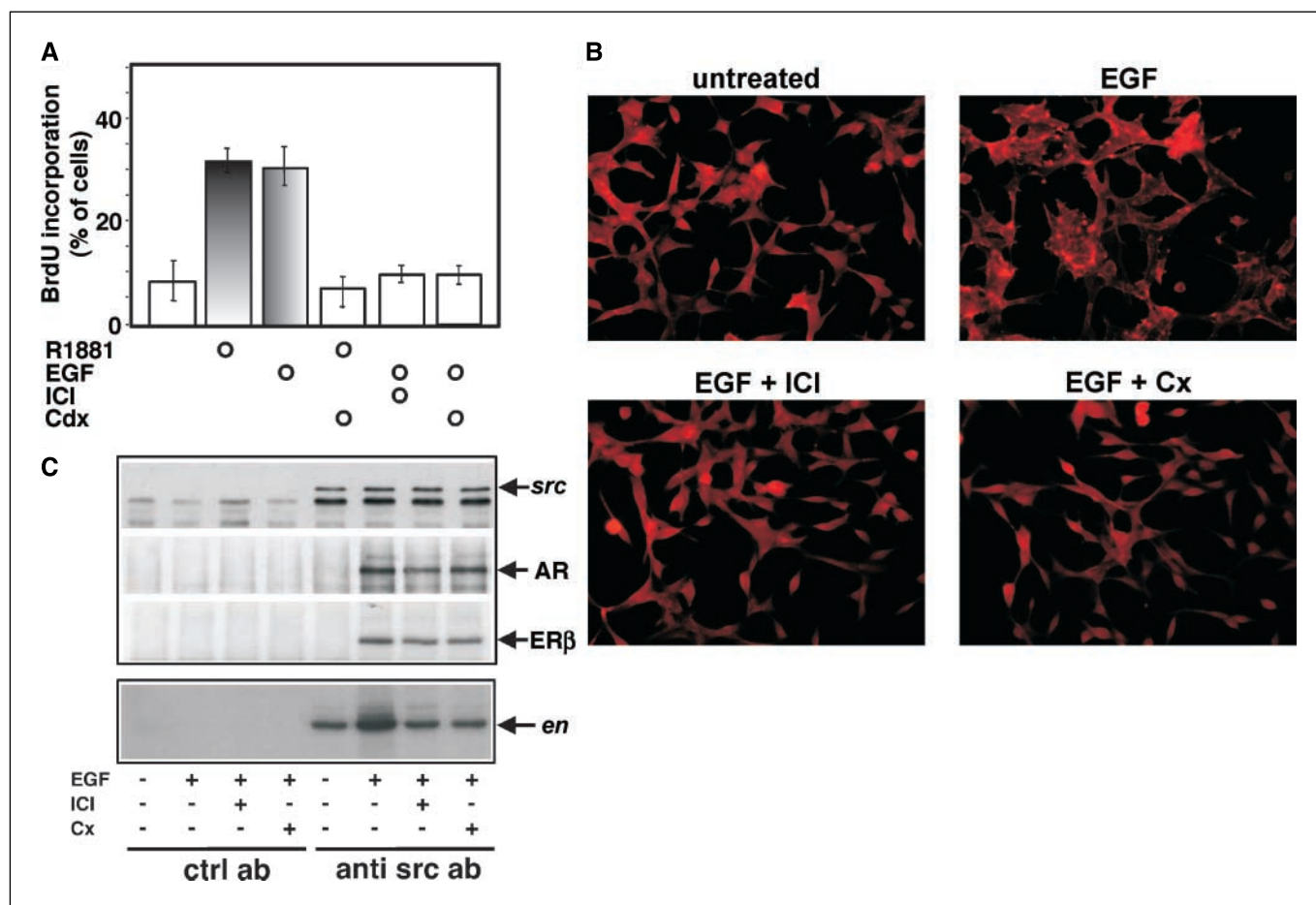


Figure 6. Steroid antagonist inhibition of the effects elicited by EGF in LNCaP cells. Quiescent LNCaP cells were used. *A*, cells were untreated or treated for 24 hours with the indicated compounds. The synthetic androgen R1881 was used at 10 nmol/L. After *in vivo* labeling with BrdUrd, DNA synthesis was analyzed and BrdUrd incorporation was expressed as a percentage of total nuclei. *Columns*, means; *bars*, SE. *B*, cells stained with Texas red phalloidin were visualized with a fluorescent microscope using a $\times 40$ lens. Micrographs are representative of three independent experiments. *C*, the cells were left unstimulated or stimulated for 5 minutes with the indicated compounds. Lysates were immunoprecipitated with either anti-Src or control antibodies. Immunoprecipitates were either blotted with antibodies against the indicated proteins or assayed for Src kinase activity using enolase as a substrate (*bottom*).

On the basis of previous and present findings, the ER/AR/Src association is crucial for proliferation triggered by steroid hormones (11) or EGF in hormone-responsive cells. This is an important point because a large number of mammary and prostate cancers respond to steroid hormones and growth factors.

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