Epidermal Growth Factor Directs Sex-specific Steroid Signaling through Src Activation*

Received for publication, November 9, 2006, and in revised form, January 16, 2007. Published, JBC Papers in Press, February 5, 2007, DOI 10.1074/jbc.M610444200

Taro Hitosugi1, Kazuki Sasaki1,2, Moritoshi Sato3, Yoshiko Suzuki, and Yoshio Umezawa4
From the Department of Chemistry, School of Science, The University of Tokyo, and Japan Science and Technology Agency, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Estrogens and androgens exert many biological effects that do not require interactions of their receptors with chromosomal DNA. However, it has been a long-standing question how the sex steroid receptors provoke signal transduction outside the nucleus. Here we have shown that epidermal growth factor (EGF) directs sex-specific steroid signaling through Src activation. We have revealed that estrogen (E2)-induced Src activation takes place in not only plasma but also endomembranes. This was found ascribed to the existence of EGF and the occurrence of EGF receptor (EGFR)-involved endocytosis of estrogen receptor together with Src. EGFR, estrogen receptor, and Src were found to form a complex upon E2 stimulation. The cell growth of breast cancer-derived MCF-7 cells was found to remarkably increase through the above EGF-involved estrogen-signaling process. In contrast, the androgen 5α-dihydrotestosterone-induced Src activation occurs only in the plasma membrane free from the interaction of EGFR with androgen receptor, irrespective of EGF. The cell growth occurred only moderately as a result. The spatial difference in Src activation between E2 and 5α-dihydrotestosterone may be responsible for the different extent of observed cell growth.

Besides traditional genomic pathways of sex steroid receptors in the nucleus, the extranuclear non-genomic pathways of these receptors are being revealed to strongly relate to many biological consequences, including vascular protection and cell proliferation (1–3). These non-genomic pathways are rapidly mediated through several critical protein kinases. A non-receptor protein tyrosine kinase, Src, is known to be activated immediately after a steroid stimulation (4, 5). This activated Src phosphorylates a wide variety of substrate proteins (such as Shc), which finally induce gene transcription (6, 7). In previous studies, the rapid activation of Src and other kinases by steroids has never been demonstrated in living cells.

To answer the question about how the Src activity is non-genomically regulated by steroid receptors in single living cells, we developed a fluorescent indicator for Src kinase activity, and named it Srcus. This indicator can monitor substrate phosphorylation by activated endogenous Src as a fluorescent resonance energy transfer (FRET)5 response in single living cells. Based on the fluorescence imaging with the present fluorescent indicators, we demonstrated that E2-induced Src activation takes place in not only plasma but also endomembranes. This was found ascribed to the existence of epidermal growth factor (EGF) and the occurrence of EGF receptor (EGFR)-involved endocytosis of estrogen receptor (ER) together with Src. EGFR, ER, and Src were found to form a complex upon E2 stimulation. The cell growth of breast cancer-derived MCF-7 cells was found to remarkably increase through the above EGF-involved estrogen-signaling process. In contrast to estrogen-activated Src signaling, the male steroid hormone 5α-dihydrotestosterone (DHT) was found to activate Src only in the plasma membrane irrespective of EGF free from the interaction of EGFR with androgen receptor (AR). The cell growth occurs only moderately as a result. The spatial difference in Src activation between E2 and DHT may be responsible for the different extent of MCF-7 cell growth between E2 and DHT.

EXPERIMENTAL PROCEDURES

Materials—CFP mutations were F64L/S65T/Y66W/N146I/M153T/V163A/N212K, and YFP mutations were S65G/V68L/Q69K/S72A/T203Y. 17β-estradiol (E2), EGF, Eagle’s minimal essential medium, phenol red-free Eagle’s minimal essential medium, and flutamide were purchased from Sigma. Fetal calf serum, Hanks’ balanced salt solution, Lipofectamine 2000 reagent, and mammalian expression vector pcDNA3.1(+) were obtained from Invitrogen. PP2 was purchased from Calbiochem. ICI 182,780 was obtained from Wako Pure Chemical Industries (Osaka, Japan). Anti-EGFR (1005G), anti-ERα (F-10), and anti-AR (441) antibodies for immunoprecipitation experiments and anti-Src rabbit polyclonal antibody for immunoblotting experiments were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Src antibody (GD11) for immunoprecipitation experiments was obtained from Upstate Biotechnology (Lake Placid, NY). Anti-GFP antibody

* This work was supported by grants from the Japan Science and Technology Agency and Japan Society for the Promotion of Science. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 These authors contributed equally to this work.

2 Present address: Chemical Genetics Laboratory, RIKEN, Wako, Saitama 351-0198, Japan

3 To whom correspondence may be addressed. Tel.: 81-3-5841-4351; Fax: 81-3-5841-8349; E-mail: sato@chem.s.u-tokyo.ac.jp.

4 To whom correspondence may be addressed. Tel.: 81-3-5841-4351; Fax: 81-3-5841-8349; E-mail: umezawa@chem.s.u-tokyo.ac.jp.

5 The abbreviations used are: FRET, fluorescent resonance energy transfer; EGF, epidermal growth factor; EGFR, EGF receptor; ER, estrogen receptor; AR, androgen receptor; CFP, cyan fluorescent protein; GFP, green fluorescent protein; YFP, yellow fluorescent protein; PTB, phosphotyrosine binding; DCC, dextran/charcoal; SH2, Src homology 2; ERK, extracellular signal-regulated kinase; TM, transmembrane.
was obtained from Clontech (Palo Alto, CA). Anti-EGFR mouse monoclonal antibody for immunoblotting experiments was obtained from BD Transduction Laboratories (San Jose, CA). Other chemicals used were all of analytical reagent grade.

**Plasmid Construction**—To construct the cDNAs encoding the present fluorescent indicators, fragment cDNAs of CFP, YFP with a nuclear export signal, a linker, and substrate domain, phosphorylation recognition domain, phosphotyrosine binding (PTB) domain, and an N-terminal transmembrane domain of Cbp were generated by standard PCR and subcloned into pBluescript (SK+). All cloning enzymes were from Takara Biomedical (Tokyo, Japan) and were used according to the manufacturer’s instructions. All PCR fragments were sequenced with ABI310 genetic analyzer.

The amino acid sequence containing the substrate and flexible linker is EEIYGEFFGNNGNNGNNGNNGNNGNNGNNGNNGNNGGNGGNG, which is reported to be selectively phosphorylated by Src (8). The phosphorylation recognition domain is an Src homology 2 (SH2) domain of Src-(148–248). The amino acid sequence of the nuclear export signal is LPPLERLTL, which is derived from a human immunodeficiency virus-derived protein, Rev. The PTB domain is derived from Shc-(46–206), and the transmembrane domain is from Cbp-(1–52). In the mutant PTB F198V domain, an amino acid residue at position 198 of Shc-(46–206) is mutated from phenylalanine to valine. All of the constructs were cloned into pcDNA3.1 (+).

**Imaging of Cells**—MCF-7 cells were treated with a medium (phenol red-free Eagle’s minimal essential medium) supplemented with dextran/charcoal (DCC)-treated serum, 1% penicillin/streptomycin, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, and 2 mM L-glutamine for 12 h followed by starvation with a serum-free medium (phenol red-free Eagle’s minimal essential medium supplemented with 1% penicillin/streptomycin, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, and 2 mM L-glutamine for 2 h. After starvation with the serum-free medium, the culture medium was replaced with Hanks’ balanced salt solution for imaging. As described previously (9), the cells were imaged at room temperature on a Carl Zeiss Axiovert 135 microscope with a cooled CCD camera MicroMAX (Roper Scientific Inc., Tucson, AZ) controlled by MetaFluor (Universal Imaging, West Chester, PA). The exposure time at 440 ± 10 nm of excitation was 100 ms. The fluorescence images were obtained through 480 ± 15- and 535 ± 12.5-nm filters with a 40× oil immersion objective (Carl Zeiss, Jena, Germany).

**Cell Culture and Transfection**—MCF-7 cells were cultured in Eagle’s minimal essential medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids at 37 °C in 5% CO₂. 12 h after transfection with Lipofectamine 2000 reagent, the MCF-7 cells were placed onto glass-bottom dishes and plastic culture dishes for fluorescence imaging of living cells and Western blotting analysis, respectively.

**Immunoprecipitation and Immunoblot Analysis**—MCF-7 cells were starved with serum-free medium for 2 h following the treatment with medium containing DCC-treated serum for 12 h. After stimulation, the cells were lysed with an ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 10 mM NaF, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml peptatin, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). After adding antibodies, the immunocomplexes were precipitated from the whole cell lysates with protein G-Sepharose 4FF beads (Amersham Biosciences). The samples were separated by SDS-PAGE, electrophoretically transferred onto polyvinylidene difluoride membrane, and probed with the appropriate antibodies. The obtained signal was quantified using an image analyzer (LAS-1000plus, Fujifilm).

**RESULTS**

**Fluorescence Indicators Visualize Rapid Src Signaling Stimulated by Sex Steroids**—The principle of Srcus is schematically shown in Fig. 1a. Upon phosphorylation of the substrate sequence by activated endogenous Src, the adjacent SH2 domain binds with this phosphorylated sequence, and FRET is induced between the two fluorescent units (10, 11). Phosphorylation-dependent FRET responses of the indicator are thus observed as a decrease in the fluorescence emission ratio of two GFP mutants. We constructed a cDNA that encodes Srcus (Fig. 1b) and introduced the cDNA in MCF-7 cells, which are derived from human female breast cancer cells and endogenously express ER and AR (5, 6). When Srcus was expressed, it was observed in the cytosol of MCF-7 cells due to a nuclear export signal sequence attached to the C terminus of Srcus (Fig. 1b). Before a steroid stimulation, the cells were made quiescent by incubation for 12 h with a medium supplemented with DCC-treated serum, as treated in previous studies on steroid signaling (4–6). Upon stimulation with 17β-estradiol (E2) of the fluorescent cell expressing Srcus, pseudocolor images of the cell that represent the CFP/YFP emission ratios of Srcus showed a blue shift in a time-dependent fashion (Fig. 1c). We next examined whether the observed FRET response of Srcus was actually caused by phosphorylation by Src in MCF-7 cells. When the tyrosine in the substrate sequence of Srcus was replaced with alanine that had no phosphoacceptor site, no significant change in the CFP/YFP emission ratio was observed upon E2 stimulation (Fig. 1d). Pretreatment of MCF-7 cells with a potent inhibitor for Src kinase activity, PP2, completely abolished the FRET response of Srcus upon E2 stimulation (Fig. 1d). Also, overexpression of Src in MCF-7 cells enhanced the response of Srcus upon E2 stimulation (Fig. 1e). These results indicate that E2- and DHT-dependent FRET responses of Srcus accurately detect the substrate phosphorylation of activated endogenous Src.

The physiological concentration of 10 nM E2 elicits the significant FRET response of Srcus (Fig. 2, a and b). This FRET response initiated soon after E2 stimulation, as shown in the time course of the change in the CFP/YFP emission ratio of Srcus (Fig. 2a). An androgen, 10 nM DHT also elicited a similar FRET response as was observed with E2 stimulation (Fig. 2, c and d). The FRET response of Srcus induced by E2 or DHT was in a concentration-dependent manner (Fig. 2, b and d). Srcus was found to show its maximum response upon stimulation of the cells with E2 and DHT at 1 μM, respectively (date not
shown). Pretreatment of MCF-7 cells with the ER antagonist ICI 182,780 completely inhibited the E2-induced FRET response of Srcus, and the AR antagonist flutamide was shown to suppress the Srcus response induced by DHT stimulation (Fig. 2e). These results indicate that the present Srcus enables to accurately monitor Src activation induced by the physiological concentration of E2 through ER and by that of DHT through AR in living cells, respectively.

**FIGURE 1.** Fluorescent indicators visualize rapid Src signaling stimulated by sex steroids. a, principle of Srcus for monitoring Src activation. CFP and YFP are different colored mutants of GFP. Upon phosphorylation of the substrate sequence within Srcus by Src, the adjacent SH2 domain binds with the phosphorylated substrate sequence, which increases the efficiency of FRET between the GFP mutants within Srcus. b, schematic representations of domain structures of Srcuses. Restriction sites are also shown with the constructs. Kz is an abbreviation of a Kozak sequence, which allows optimal translation initiation in mammalian cells. The amino acid sequence of the substrate sequence is EEEIYGEFF, which is preferentially phosphorylated by Src. The SH2 domain is derived from Src-(148–248). Ln is an abbreviation of a flexible linker sequence, GGNNNGNNNGNNGNNGNNNGNGNGNGNG. nes, of which amino acid sequence is LPPL-ERLTL, is an abbreviation of a nuclear-export-signal sequence derived from the human immunodeficiency virus protein Rev. The PTB domain is derived from Shc-(46–206). The transmembrane domain is from Cbp-(1–52). In the mutant PTB F198V domain, an amino acid residue at position 198 of Shc-(46–206) is mutated from phenylalanine to valine. c, pseudocolor images of the MCF-7 cells expressing Srcus. The emission ratio change of CFP to YFP is drawn as the pseudocolor change. The data are obtained before (time 0 s) and at 10 and 20 min after the addition of 1 μM E2. The MCF-7 cells were pretreated overnight with the medium containing DCC-treated serum to make the cells quiescent. d, a comparison of the Srcus response with the response of Srcus-A upon 1 μM E2 stimulation and with the 1 μM E2-induced response of Srcus by pretreating MCF-7 cells with 5 nM PP2 for 1 h. The results are means ± S.D. of three independent experiments. e, an enhanced response of Srcus in MCF-7-Src cells compared with the Srcus response in MCF-7 cells upon 1 μM E2 stimulation. The results are means ± S.D. of three independent experiments.
EGF Is Required for Estrogen-dependent Src Activation but Not for Androgen—The DCC-treated serum used in the starvation of steroids is known to contain polypeptide growth factors (12). To rule out the possibility that these serum-derived factors may regulate the non-genomic Src activation, we treated the MCF-7 cells overnight with the medium containing DCC-treated serum followed by the starvation of the cells with a serum-free medium for 2 h. To examine the inhibitory effect of...
with E2 alone or together with EGF under serum-free medium. The EGF or E2 stimulation alone in the serum-free medium showed little proliferative effect on MCF-7 cell growth (Fig. 3e, open bar and bar with black stripes). However, the co-stimulation of MCF-7 cells with E2, together with EGF, remarkably promoted MCF-7 cell growth (Fig. 3e, gray bar). DHT stimulation of the cells in the serum-free medium only moderately promoted MCF-7 cell growth irrespective of EGF (Fig. 3e, black and gray bars with white dots). These results clearly indicate that not only E2 but also EGF is required for estrogen-dependent MCF-7 cell growth.

The Difference between Estrogen and Androgen in EGF Dependence of Src Activation Is because of the Binding of EGFR Only with ER but Not with AR—As shown in Fig. 3, we found that the estrogen-induced Src activation requires EGF, but the androgen-induced Src activation does not require EGF. To reveal the molecular basis of this difference in EGF dependence of Src activation between estrogen and androgen, the immunoprecipitates with the anti-EGFR antibody were immunoblotted by anti-ERα antibody. After the starvation of the cells with the medium containing DCC-treated serum for 12 h, the cells were treated with the serum-free medium for 2 h and then stimulated with E2 or DHT at 1 μM for 15 min to perform the experiments under the same condition as Fig. 3, a and b. ERα was found to stably bind with EGFR irrespective of E2 and EGF stimulations (Fig. 4a). Furthermore, we performed the immunoblotting of anti-Src immunoprecipitates with anti-ERα and -EGFR antibodies. Src was found to bind with ERα upon stimulation of the cells with E2 alone or with both E2 and EGF (Fig. 4b). EGFR was bound to immunoprecipitates of Src when the cells were separately or simultaneously stimulated with E2 and EGF (Fig. 4b). These results indicate that EGFR, ERα, and Src form a complex in an E2-dependent manner.

To determine whether the activated EGFR-ERα complex interacts with Src for the Src activation, we let Srcus be accessible to the activated EGFR-ERα complex by fusing a PTB domain of Shc to the N terminus of Srcus and named it PTB-Srcus (Fig. 1b). This PTB domain is known to bind with a cytoplasmic domain of EGF-activated EGFR (13, 14). If the activated EGFR-ERα complex interacts with Src for the Src activation, PTB-Srcus would be more efficiently phosphorylated by activated Src through this complex than the mutated PTB-Srcus (mPTB-Srcus) that has a mutant PTB F198V domain, which does not bind with EGFR (15) (Fig. 1b). As shown in Fig. 4c, when the MCF-7 cells treated with the serum-free medium were co-stimulated with E2 and EGF, the extent of phosphorylation in PTB-Srcus was twice greater than that in mPTB-Srcus. This result explains that the requirement of EGF for estrogen-dependent Src activation is because of the binding of the EGFR-ERα complex with Src. In contrast, AR exhibited no significant interaction with EGFR when cellular proteins were immunoprecipitated with an anti-AR antibody (Fig. 4d). In addition, PTB-Srcus and mPTB-Srcus were phosphorylated by Src nearly to the same extent in the DHT-stimulated cells (Fig. 4e). These results confirm that AR activates Src free from the interaction with EGFR.

To assess the interaction of ERα-EGFR complex, we depleted ERα and EGFR from MCF-7 cell lysate by using anti-ERα and...
Src-dependent Steroid Signaling Regulated by EGF

Figure a: Bar graph showing the decrease in emission ratio change (CFP/YFP) for E2 and DHT in medium containing DCC-treated serum compared to serum-free medium.

Figure b: Bar graph showing the decrease in emission ratio change (CFP/YFP) for E2 in different concentrations of EGF (0, 0.05, 0.5, 5, 50 ng/mL).

Figure c: Bar graph showing the decrease in emission ratio change (CFP/YFP) for DCC-treated serum, DCC-treated serum with anti-EGF antibody, and AG1478 in medium containing DCC-treated serum.

Figure d: Western blot analysis for EGF with antibodies against anti-pERK and anti-ERK, showing the expression levels of ERK1 and ERK2.

Figure e: Bar graph showing the increase in cell number (fold) for E2 and DHT in medium containing DCC-treated serum and serum-free medium.
-EGFR antibodies, respectively. The depleted cell lysates were immunoblotted with anti-ERα and -EGFR, respectively (Fig. 4f). We quantified that anti-EGFR immunodepletion removed EGFR completely and removed 40% of ERα from the lysate. On the other hand, 80% of ERα was removed with the complete depletion of ERα using anti-ERα antibody. These results indicate that 40% of the total ERα is bound to EGFR and 80% of the total EGFR is bound to ERα in MCF-7 cells.

E2- and DHT-induced Src Activations Occur Respectively in Spatially Different Membranes because of the Endocytosis of EGFR That Binds Only with ER but Not with AR—When the cells expressing Srcus were stimulated with the same concentration of estrogen and androgen, respectively, the emission ratio of Srcus was decreased to the same extent (Fig. 2, b and d). This result indicates that no difference between E2 and DHT was observed to the extent of Src activation in the cell. The difference in the amount of MCF-7 cell growth was, however, observed between them; E2 together with EGF remarkably increases MCF-7 cell growth, but DHT only modestly increases MCF-7 cell growth irrespective of EGF (Fig. 3e). Src exhibits a broad distribution in subcellular membranes including the plasma membrane and endomembranes, i.e. the ER and Golgi membranes (16). To examine in which membranes non-genomic Src activation is induced by ER that binds with EGFR or by AR that does not bind with EGFR, Srcus was tethered to cytoplasmic surfaces of subcellular membranes by connecting a transmembrane domain of Cbp protein (17). This membrane tethering prevents a free diffusion of the indicator and avoids the resulting loss of spatial information as to phosphorylation by the activated Src (Fig. 1b). This Srcus variant was named transmembrane (TM)-Srcus. The MCF-7 cells expressing TM-Srcus were treated with the medium containing DCC-treated serum and then stimulated separately with E2 or DHT. TM-Srcus responses therefrom in the plasma membrane and also those in the endomembranes were respectively monitored (Fig. 5, a and c). Upon E2 stimulation, the pseudocolor that represents the CFP/YFP emission ratio of TM-Srcus was changed from red to green in both plasma and endomembranes (Fig. 5a). From a time course of the CFP/YFP emission ratio, the FRET response of TM-Srcus was immediately observed in the plasma membrane, and it reached a plateau (Fig. 5b). In the endomembranes, E2 gradually elicited the TM-Srcus response (Fig. 5b). Src was thus activated in both plasma and endomembranes upon E2 stimulation. With DHT stimulation, however, the pseudocolor was changed from red to green only in the plasma membrane (Fig. 5c); no significant response of Srcus was detected in the endomembranes (Fig. 5d).

The observed spatial difference in Src activation between ER and AR may be due to dissimilar involvement of EGFR in ER and AR; ER was found to bind with EGFR, and AR does not bind with EGFR (see above). EGFR is known to be transported from the plasma membrane to the endomembranes by its endocytosis. The endocytosis of EGFR is inhibited by a dominant negative dynamin mutant (DynK44A) (9, 19). We overexpressed DynK44A in MCF-7 cells and monitored the response of TM-Srcus in the endomembranes, which completely inhibited the E2-induced response of TM-Srcus in the endomembranes (Fig. 5, b and e). However, no such effect was observed in the Src activation in the plasma membrane (Fig. 5, b and e). These results indicate that E2-activated Src is transported from the plasma membrane to the endomembranes as the Src-ER-EGFR complex by the EGFR endocytosis. In contrast, DHT-activated Src, which does not interact with EGFR, was found not to be transported to the endomembranes. To conclude, in the plasma membrane, both estrogen- and androgen-induced Src activations take place, respectively, but in the endomembranes, only estrogen-induced Src activation is elicited by the endocytosis of EGFR that binds with ER and not with AR. This Src activation in the endomembranes may probably explain the difference in the extent of the observed MCF-7 cell growth between estrogen and androgen (Fig. 3e).

**DISCUSSION**

In the present study, we have found that estrogen alone is not sufficient to rapidly activate Src. We have shown that not estrogen alone but rather co-stimulation with estrogen and EGF is required for the rapid Src activation. In contrast, androgen was found to activate Src without the EGF stimulation. In addition, we have shown that not only E2 but also EGF is required for estrogen-dependent MCF-7 cell growth, although EGF is not required for estrogen-dependent Src activation and MCF-7 cell growth but not for androgen. a, effects of the serum-free medium starvation on the Srcus response in the MCF-7 cells stimulated with E2 and DHT at 1 μM for 15 min. Open bars represent the Srcus response when the cells were pretreated with the medium containing DCC-treated serum for 12 h. Closed bars represent the Srcus response when the cells were pretreated with the serum-free medium for 2 h following the treatment of the cells with the medium containing DCC-treated serum for 12 h. The results are means ± S.D. of three independent experiments (mean ± S.D.). b, Src is activated in a non-genomic fashion by co-stimulation with E2 and EGF. The open bar represents the Srcus response for 1 μM E2 stimulation on MCF-7 cells, which were pretreated with the medium containing DCC-treated serum for 12 h. Closed bars represent the Srcus response for each different concentration of EGF stimulation together with and without 1 μM E2 stimulation on the cells, which were pretreated with the serum-free medium for 2 h following the treatment of the cells with the medium containing DCC-treated serum for 12 h. The results are means ± S.D. of three independent experiments. c, effects of EGF and its receptor activation on E2-induced Src activation in the cells incubated with the DCC-treated serum. The open bar represents the Srcus response for 1 μM E2 stimulation in MCF-7 cells, which were pretreated with the medium containing DCC-treated serum for 12 h. The closed bar represents the Srcus response for 1 μM E2 stimulation on MCF-7 cells, which were pretreated for 12 h with the medium containing DCC-treated serum for 10 μg/ml anti-EGFR antibody. The gray bar represents the Srcus response for 1 μM E2 stimulation in MCF-7 cells, which were pretreated for 30 min with 100 μM AG1478 after incubation of the cells for 12 h with the medium containing DCC-treated serum. The results are means ± S.D. of three independent experiments. d, ERK activation is induced by EGFR in the cells treated with the medium containing DCC-treated serum. After starvation of the cells for 12 h with the medium containing DCC-treated serum, the starved cells were further incubated for 2 h in the serum-free medium and then stimulated with 100 ng/ml EGF for 15 min. E8, immunoblot. e, role of EGF for estrogen-dependent MCF-7 cell growth. The MCF-7 cells were stimulated with 10 nM E2 or 2 μg/ml EGF under a serum-free medium (open bar and bar with black stripes). The co-stimulation of the cells with E2 and EGF under the serum-free medium promotes cell growth (gray bar). We also stimulated the MCF-7 cells with 100 nM DHT alone or together with 2 μg/ml EGF under the serum-free medium (black and gray bars with white dots). The MCF-7 cells thus treated were incubated at 37 °C for 5 days. The numbers of respectively treated MCF-7 cells were counted. The number of the MCF-7 cells maintained for 5 days under the steroid-free medium with or without the 10 nM E2 stimulation was also shown as a control of MCF-7 cell growth (bar with black dots and bar with black wavelike lines). The results are means ± S.D. of three independent experiments.

T. Hitosugi, M. Sato, K. Sasaki, and Y. Umezawa, submitted for publication.
Src-dependent Steroid Signaling Regulated by EGF

The results are means of three independent experiments. The extent of phosphorylation of PTB-Srcus and mPTB-Srcus were normalized with that of Srcus. The results are means of three independent experiments. The extent of phosphorylation of PTB-Srcus and mPTB-Srcus were normalized with that of Srcus. The results are means of three independent experiments.

**FIGURE 4.** The difference between E2 and DHT in EGF dependence of Src activation is due to the binding of EGFR with ER and not with AR. a, the immunoprecipitated cellular proteins with the anti-EGFR antibody were probed with anti-ERα or -EGFR antibodies. The MCF-7 cells starved with the serum-free medium were separately or simultaneously stimulated with 1 μM E2 and 50 ng/ml EGF. b, E2-dependent complexation of EGFR, ERα, and Src. The MCF-7 cells starved with the serum-free medium were separately or simultaneously stimulated with 1 μM E2 and 50 ng/ml EGF. The cell lysates were immunoprecipitated with the anti-Src antibody. The immunoprecipitates were separately probed with anti-ERα, -EGFR, and -Src antibodies. c, a comparison of the responses between Srcus, PTB-Srcus, and mPTB-Srcus upon co-stimulation with 1 μM E2 and 5 μg/ml EGF. The extent of phosphorylation of PTB-Srcus and mPTB-Srcus were normalized with that of Srcus. The results are means of three independent experiments. d, the immunoprecipitated cellular proteins with the anti-ERα or -AR antibody were probed with the anti-AR and -EGFR antibodies. AR exhibited no significant interaction with EGFR regardless of DHT stimulation. e, a comparison of the responses between Srcus, PTB-Srcus, and mPTB-Srcus upon co-stimulation with 1 μM E2 and 5 μg/ml EGF. The extent of phosphorylation of PTB-Srcus and mPTB-Srcus were normalized with that of Srcus. The results are means of three independent experiments. f, the interaction of ERα-EGFR complex in MCF-7 cells. The MCF-7 cells in the culture medium were lysed. The cell lysate was incubated overnight with anti-ERα and -EGFR antibody to deplete ERα and EGFR, respectively, and for 2 h with protein-Sepharose beads. After incubation, anti-ERα or -EGFR immunoprecipitates were removed by centrifugation, and the supernatants were collected as a sample. The collected samples were immunoblotted (IB) with anti-ERα and -EGFR antibody, respectively.

necessary for androgen-dependent cell growth. Prior to the present research, it has long been believed that the female steroid hormone estrogen rapidly induces signal transduction, such as Src activation, by itself and subsequently shows its proliferative effects on cells. However, in these earlier works, the cells have been stimulated with estrogen in the presence of DCC-treated serum that still contains many kinds of factors such as EGF. Because of this experimental problem, the present key factor EGF has not been found for the rapid estrogen signaling in previous studies. We have further evidenced how Src is synergistically activated by the co-stimulation with E2 and EGF. ERα was found to stably bind to membrane-spanning EGFR, and this ERα-EGFR complex bound to Src in an E2-dependent fashion. On the other hand, AR was found to activate Src without the interaction of EGFR. These findings provide the explanation at the molecular level for the requirement of EGF exclusively in estrogen-dependent Src activation, but this is not the case for androgen.

The substrate sequence in Srcus is the optimized peptide as a substrate for Src and is poorly phosphorylated by Abl, one of the other tyrosine kinases (8). In addition, as shown in Fig. 1, the E2-induced FRET response of Srcus is completely blocked by PP2 and is enhanced with the overexpression of Src in MCF-7 cells. However, we should note the possibility that Srcus may report activation of other members of the Src family kinases, as PP2 also inhibits the activity of other Src family kinases.

From the responses of Srcus induced by the same concentration of estrogen and androgen, no difference was observed in the extent of Src activation in the cell. The difference in the amount of MCF-7 cell growth was, however, observed between them; E2 together with EGF remarkably increased MCF-7 cell growth, but DHT only moderately increased MCF-7 cell growth irrespective of EGF. Although we knew that both estrogen and androgen non-genomically activates Src, we did not know how estrogen and androgen exhibit the difference in the physiological effects involving activated Src. From the fluorescence imaging with TM-Srcus, we have shown the difference in the subcellular location of Src activation between E2 and DHT. E2 was found to activate Src in not only plasma but also endomembranes. We found that this Src activation in the plasma and endomembranes requires EGF and is regulated by the occurrence of EGFR-involved endocytosis of ER together with Src. In contrast to estrogen-activated Src signaling, the male steroid hormone DHT was found to activate Src only in the plasma membrane irrespective of EGF free from the interaction of EGFR with AR. The spatial difference in Src activation induced by E2 or DHT may be responsible for the different extent of MCF-7 cell growth between E2 and DHT.

We have found that EGF plays a requisite role in estrogen-dependent MCF-7 cell growth. EGF knock-out mice have pre-
FIGURE 5. E2- and DHT-induced Src activations occurred, respectively, in spatially different membranes due to the endocytosis of EGFR that binds with ER and not with AR. a, left, pseudocolor image of the cells expressing TM-Srcus before E2 stimulation. The region enclosed by white lines represents the plasma membrane of a single MCF-7 cell. The region enclosed by white dotted lines represents a part of the endomembranes. a, right, pseudocolor image of the cells stimulated with 1 μM E2 for 10 min. The region enclosed by white lines represents the plasma membrane of a single MCF-7 cell. The region enclosed by white dotted lines represents a part of the endomembranes. b, time courses of the TM-Srcus response upon 1 μM E2 stimulation in the regions of the plasma membrane and endomembranes. c, left, pseudocolor image of the cells expressing TM-Srcus before DHT stimulation. The region enclosed by white lines represents the plasma membrane of a single MCF-7 cell. The region enclosed by white dotted lines represents a part of the endomembranes. c, right, pseudocolor image of the cells stimulated with 1 μM DHT for 10 min. The region enclosed by white lines represents the plasma membrane of a single MCF-7 cell. The region enclosed by white dotted lines represents a part of the endomembranes. d, time courses of the TM-Srcus response upon 1 μM DHT stimulation in the regions of the plasma membrane and endomembranes. e, by overexpression of DynK44A, the TM-Srcus response upon E2 at the endomembranes was completely inhibited to the basal level, but the response at the plasma membrane was not affected. In a–e, the MCF-7 cells were treated with the medium containing DCC-treated serum for 12 h before the fluorescence imaging studies. The cells were stimulated at time 0 s. The results shown in a–e represent typical experiments from three independent measurements.
viously been reported to show impaired growth and an impaired proliferative response to E2 stimulation in stromal cells of uterine and vaginal grafts (20). This report appears to suggest a clear link between EGFR and estrogen signaling in vivo, although other tissues and organs of the EGFR knockout mice have not been dissected in regard to this point. Our finding on the interaction of EGFR with ER for Src activation provides a clear explanation at the molecular level for the above mentioned in vivo observation of the EGFR knockout mice.

In an earlier work, ~20% of E2 binding sites have been observed in the plasma membrane of MCF-7 cells, and the other sites include the nucleus (45%), cytosol (10%), and other organelles (2). In addition, membrane-impermeable conjugates of E2 and bovine serum albumin (E2-BSA) have evoked downstream signals, including the Src activation likewise membrane-permeable E2 (21, 22). From these earlier observations, ER that non-genomically activates the downstream signals has been believed to locate at the plasma membrane. Several groups have actually addressed this issue. Levin and co-workers (23) have reported the membrane localization of ER through the interaction with cavelolin. Santen and co-workers (24) have described a role of truncated forms of ER on the membranes (25). Our present results provide a molecular basis that correlates the association of ER with membrane-spanning EGFR and explains how ER locates at the plasma membrane and non-genomically transmits the female steroid signal to the Src activation.

From a clinical viewpoint, overexpression of both the ER and erbB family, such as EGFR/erbB1, HER2/erbB2, and erbB3, are well known to be important prognostic factors in human breast cancer (18, 26). We herein have found that EGFR, ER, and Src form the complex in an estrogen-dependent manner, and the activation of both ER and EGFR is required for estrogen-dependent Src activation and cell growth of human breast cancer-derived MCF-7 cells. In other words, the ER-EGFR-Src complex probably plays an important role in the tumorigenesis occurring in human breast tissues.

Acknowledgment—We thank Y. Imai for experimental help.

REFERENCES
1. Losel, R., and Wehling, M. (2003) Nat. Rev. Mol. Cell Biol. 4, 46–56
2. Marquez, D. C., and Pietras, R. J. (2001) Oncogene 20, 5420–5430
3. Kousteni, S., Chen, J.-R., Bellido, T., Han, L., Ali, A. A., O’Brien, C. A., Plotkin, L., Fu, Q., Mancino, A. T., Wen, Y., Vertino, A. M., Powers, C. C., Stewart, S. A., Ebert, R., Parfitt, A. M., Weinstein, R. S., Jilka, R. L., and Manolas, S. C. (2002) Science 298, 843–846
4. Migliaccio, A., Castoria, G., Di Domenico, M., de Falco, A., Bilancio, A., Lombardi, M., Barone, M. V., Ametrano, D., Zannini, M. S., Abbondanza, C., and Auricchio, F. (2000) EMBO J. 19, 5406–5417
5. Castoria, G., Migliaccio, A., Bilancio, A., Di Domenico, M., de Falco, A., Lombardi, M., Fiorentino, R., Varricchio, L., Barone, M. V., and Auricchio, F. (2001) EMBO J. 20, 6050–6059
6. Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E., and Auricchio, F. (1996) EMBO J. 15, 1292–1300
7. Kousteni, S., Bellido, T., Plotkin, L. I., O’Brien, C. A., Bodenner, D. L., Han, L., Han, K., DiGregorio, G. B., Katzenellenbogen, J. A., Katzenellenbogen, B. S., Roberson, P. K., Weinstein, R. S., Jilka, R. L., and Manolas, S. C. (2001) Cell 104, 719–730
8. Songyang, Z., Carraway, K. L., Eck, M. J., Harrison, S. C., Feldman, R. A., Mohammadi, M., Schlessinger, J., Hubbard, S. R., Smith, D. P., Eng, C., Lorenzo, M. J., Ponder, B. A. J., Mayer, B. J., and Cantley, L. C. (1995) Nature 373, 536–539
9. Sato, M., Ueda, Y., Takagi, T., and Umezawa, Y. (2003) Nat. Cell Biol. 5, 1016–1022
10. Sato, M., Ozawa, T., Inukai, K., Asano, T., and Umezawa, Y. (2002) Nat. Biotechnol. 20, 287–294
11. Wang, Y., Botvinick, E. L., Zhao, Y., Berns, M. W., Usami, S., Tsiens, R. Y., and Chien, S. (2005) Nature 434, 1040–1045
12. van Zoelen, E. J., van Oostwaard, T. M., van der Saag, P. T., and de Laat, S. W. (1985) J. Cell. Physiol. 123, 151–160
13. Olayioye, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000) EMBO J. 19, 3159–3167
14. Wolf, G., Trub, T., Ottinger, E., Groninga, L., Lynch, A., White, M. F., Miyazaki, M., Lee, J., and Shoelson, S. E. (1995) J. Biol. Chem. 270, 27407–27410
15. Yajnik, V., Blakie, P., Bork, P., and Margolis, B. (1996) J. Biol. Chem. 271, 1813–1816
16. Kawakatsu, H., Sakai, T., Takagaki, Y., Shinoda, Y., Saito, M., Owada, M. K., and Yano, J. (1996) J. Biol. Chem. 271, 5680–5685
17. Kawabuchi, M., Satomi, Y., Takao, T., Shimonishi, Y., Nada, S., Nagai, K., Takeda, H., and Okada, M. (2000) Nature 404, 999–1003
18. de Bono, J. S., and Rowinsky, E. K. (2002) Trends Mol. Med. 8, S19–S26
19. Vieira, A. V., Lamaze, C., and Schmid, S. L. (1996) Science 274, 2086–2089
20. Hom, Y. K., Young, P., Wiesen, J. F., Miettinen, P. J., Derynck, R., Werb, Z., and Cunha, G. R. (1998) J. Biol. Chem. 273, 187–197
21. Zheng, J., Ali, A., and Ramirez, V. D. (1996) J. Psychiatry Neurosci. 21, 536–539
22. Russel, K. S., Haynes, M. P., Sinha, D., Clerisme, E., and Bender, J. R. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5930–5935
23. Razandi, M., Oh, P., Pedram, A., Schnitzer, J., and Levin, E. R. (2002) Mol. Endocrinol. 16, 100–115
24. Song, R. X., Barnes, C. J., Zhang, Z., Bao, Y., Kumar, R., and Santen, R. J. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 2076–2081
25. Li, L., Haynes, M. P., and Bender, J. R. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 4807–4812
26. McGuire, W., and Clark, G. (1992) N. Engl. J. Med. 326, 1756–1762