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13. ABSTRACT (Maximum 200 Words)

Estrogen receptor α, (ERα), the dominant isoform in mammary gland, is a dual functional protein capable of acting as a transcriptional factor regulating gene transcription in the nucleus (genomic effect), or a signaling molecule on the membrane mediating estrogen rapid actions (non-genomic effect) to activate MAPK and Akt pathways. Currently, the functions of the nuclear ERα is very well studied, but the biological role of the membrane-associated ERα is still not clear due to the fact that all ERα-expressing cells have both nuclear and membrane ones co-expressed. One way to investigate the biological role of the membrane-associated ERα in breast cancer cells is to develop a cell line that only express the ERα on the cell membrane or in the cytosol (Designer cells). The goal of this proposal is to develop designer COS-1 cells, which lack endogenous ERα, with constructs that express forms of the ERα that are specifically cytosolic or membrane-associated, and use these tools in experiments designed to elucidate the functional role(s) of the membrane-associated, or cytosol ERα, especially its role in interacting and activating Shc adapter protein, leading to MAP kinase activation.

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INTRODUCTION

Estrogen, of which 17β-estradiol (E2) constitutes the major endogenous form, has been confirmed unambiguously to promote breast cancer in post-menopausal women by results of the Women's Health Initiative [1]. Its action is mediated by its receptor α and β (ERα and β). ERα, the dominant isoform in mammary gland, is a dual functional protein capable of acting as a transcriptional factor regulating gene transcription in the nucleus (genomic effect), or a signaling molecule on the membrane mediating estrogen rapid actions (non-genomic effect) to activate MAPK and Akt pathways. The existence of a cell membrane-associated ERα has been widely accepted, but its biological role on the regulation of cell proliferation and apoptosis is still controversial. Furthermore, the ERα-mediated membrane actions occur within seconds to minutes, making the involvement of transcriptional events unlikely. The biological effects of nuclear ERα on cell growth and proliferation are very well studied, but the role of the membrane-associated ERα in mediating estrogen non-genomic actions are largely unknown.

Current therapy for controlling the growth of ER-positive breast cancer is primarily based on the strategy of interrupting nuclear ERα-mediated transcription, and is hampered by lack of knowledge of the molecular basis for the membrane-associated ERα functions and for it role in regulation of its downstream kinase cascades.

The great challenge in studying estrogen-induced cell membrane effects on cell growth is to (A) distinguish the roles of the membrane ERα from the nuclear one in endogenous ERα-expressing cells and (B) decipher the upstream signing pathways leading to E2-induced MAPK and Akt activation. The membrane-associated ERα has been demonstrated by us and others [2,3], but the study on its biological role is hampered due to the lack of a cell model only expressing the membrane ERα. A large body of the indirect evidence links the proliferative and anti-apoptotic effects of estrogen to the membrane ERα in human breast cancer, bone and other cells [4,5]. However, ERα, unlike a cell membrane growth factor receptor, is not a membrane protein due to the lack of transmembrane domains. Meantime it has no intrinsic kinase domain and cannot phosphorylate any signaling molecule. How ERα acts as a membrane estrogen receptor to transduce estrogen signals to downstream cascade and activates MAPK? To investigate the non-genomic action of estrogen, I initiated the current project a few years ago. The purpose of this project is to study the biological role of the membrane-associated ERα in response to estrogen on MAPK activation, cell proliferation and apoptosis.

In order to discriminate the functional role of the membrane ERα in estrogen rapid action, it is extremely valuable to be able to test the biological effects of the membrane ERα without the confounding effects of the nuclear one. Various efforts to study only the membrane-associated ERα have failed or presented limitations to their usefulness. For example, an effort was made to anchor ERα action at the cell membrane by preventing E2 from penetrating further than the membrane as a result of conjugation to a molecule too large to pass through (bovine serum albumin), but upon exposure to cells, free E2 leaking from the conjugate is unavoidable [6]. In another approach 4-estren-3alpha,17beta-diol, an E2-derived synthetic ligand of ERα, attracted interest because initially it was reported only to act on the membrane ERα without affecting nuclear ERα-mediated gene transcription, but later it was demonstrated to activate the estrogen-response element (ERE)-driven reporter gene at the same magnitude as E2 [7,8]. Alternatively, generation of "designer cells", which express forms of the ERα selectively in either cytosol or on the cell membrane, is anther choice to study the membrane-associated ERα without being interfered by the nuclear one.

The scope of my research includes the following specific Tasks: **(1)** construct a cell membrane (HE241G-mem), a cytosol-targeted (HE241G), and a wild type (HEGO) ERα expression vectors. We will individually transfect HE241G-mem, HE241G or HEGO into COS-1 monkey kidney epithelial

cells that has no endogenous ERα expression (designer cells). These designer cells will express above ERα in selective locations of COS-1 cells, such as either in the cytosolic, nuclear or on the membrane. **(2)** Determine the ERα location in the designer cells. **(3)** Testing the functional role of the membrane-targeted, cytosol and nuclear ERα for their response to estrogen on MAPK phosphorylation, ERE promoter activation and cell growth. **(4)** Determine the Shc-interacting domain(s) on ERα. Meantime, we also plan to study if the interruption of ERα and Shc association will affect estrogen-induced MAPK activation.

BODY AND KEY RESEARCH ACCOMPLISHMENTS

For better understanding the work progress, I combined the body and key research accomplishments in the same section and outline the report following the order of Statement of Work in my proposal:

Finished tasks:

The tasks of 1, and 2 have been fully finished in my laboratory and the results were published. Part of tasks 3 and 4 have been generated and published. The detailed information regarding the accomplishments for the tasks in my proposal as following:

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS [9] – Because estrogen rapid effects are believed to be initiated by a membrane-associated ERα, the designer cells were development by constructing several ERα expressing vectors using pSG5 plasmid: a wild type ERα (HEGO), a cytosol expressing ERα (HE241G) due to the deletion of the nuclear localization signal (NLS) of the ERα and a membrane expressing ERα (HE241G-mem), in which a membrane localization signal sequence from GAP-43 protein is expressed on the C-termini of HE241G (**Task 1a, 1b, 1c and 1d**). Expressing these vectors individually in ERα-null COS-1 cells (**Task 3a**), we demonstrated:

1. HEGO is almost exclusively expressed in the nucleus, HE241G in cytosol. HE241G-mem ERα is expressed in both cell membrane (34%) and cytosol (66%) (**Task 2a**).

2. Only HE241G-mem, but not HE241G and HEGO, mediates E2 on MAPK activation, indicating that the membrane association of ERα is required for E2-induced MAPK activation (**Task 3b**).

3. Compared with wt ERα (HEGO), ERα of HE241G-mem and HE241G did not mediate E2 effect on ERE promoter activation, further demonstrating that it is excluded from the nucleus (**Task 2b and 3c**).

Task 2b requires further justification. The purpose of this task is to test if the exogenous ERα mutants (our membrane and cytosol constructs) are exclusively expressed outside the nucleus by ERE promoter activation assay, ligand binding assay after cell fractionation and gel shift experiment. The last two approaches were designed to provide further information if the ERE promoter assay did not yield satisfaction results. However, our results from ERE promoter assay (the most sensitive assay) demonstrated that both membrane and cytosol ERα are exclusively expressed outside the cell nucleus (no luciferase induction after estrogen treatment in our designer cells), so the ligand binding and gel shift experiments are not necessary to be conducted.

MOLECULE ENDOCRINOLOGY (Cover Figure) [3] – Working on human MCF-7 breast cancer and COS-1 cells, we demonstrated:

1. ERα under E2 treatment rapidly associated with adapter protein Shc and rapidly accumulated in the area of the plasma membrane.

2. A physical interaction between ERα and Shc was further demonstrated by protein pulldown assay in COS-1 cells transfected with ERα and Shc expression vectors. N-terminal of ERα directly interacts with either PTB or SH2 domain of Shc (**Task 4a and 4c**). The pY-537 of ERα is not involved in interaction with Shc.

3. E2 rapidly induced MAPK activation assayed by measuring MAPK phosphorylation and Elk-1 transcriptional factor activation. Expression of Shc mutant or treatment of cells with anti-estrogen ICI and PP2 all blocked E2-induced MAPK activation (**Task 4d**). ICI blocked E2-induced ERα dynamic membrane translocation. Above evidence suggest that the membrane-associated ERα, Shc and Src are all upstream signals on estradiol-induced MAPK activation.

PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES [10] – This study was not proposed in my statement of work, but a continue study of task 4 of the current proposal. Based on our previous evidence that a Shc/ERα association and ERα membrane translocation were required for E2-induced MAPK activation in MCF-7 cells [3], we postulated that ERα membrane association might involve a membrane growth factor receptor, which is known to contribute—though it is still not known how—to the biological consequences of E2 exposure. Focusing on the IGF-1 receptor (IGF-1R), we have observed in breast cancer cells (MCF-7) that:

1. Upon three minutes of E2 exposure, a complex was detectable that included ERα, Shc, and IGF-1R and the IGF-1R was activated.

2. The presence of the E2-induced association of ERα/Shc and ERα/IGF-1R were observed by confocal microscopy to show their co-localization on the cell membrane, which disappeared upon downregulation of Shc or IGF-1R

3. Shc acts as a chaperon bringing ERα to the membrane by binding to IGF-1R Knockdown of either Shc or IGF-1R with selective siRNA abolished ERα translocation to the membrane region of MCF-7 cells.

4. Downregulation studies showed that ERα, Shc, and IGF-1R roles are all upstream of MAPK activation and cell proliferation in E2-induced MCF-7 breast cancer cells.

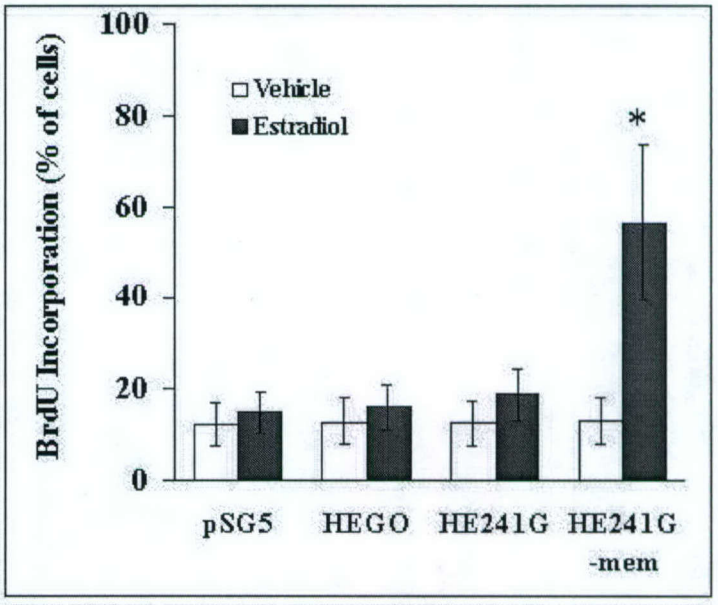

Fig. 1. Effect of estrogen on cell proliferation in designer cells stably transfected with either wt, cytosol or membrane-associated ERα .

Finished Task, but not published:

Task 3c is partial finished. In this task, I proposed to study (A) ERE promoter activation in our designer cells, (B) the effect of estrogen on cell growth by measuring cellular DNA synthesis by BrdU method or cell growth by cell number counting in our designer cells expressing the membrane-targeted, cytosol or nuclear ERα. The ERE promoter activation study has been done and published [9]. The study of estrogen effect on cell growth in our designer cells was just finished. As shown in **Fig. 1**, E2 only stimulated the cell proliferation assayed by BrdU incorporation into cellular DNA in the membrane ERα transfected cells, but not in wt and cytosol ERα transfected cells. The data indicated that the ERα expression on the cell membrane is functionally involved in E2 biological action.

Unfinished task:

Task 4b is to determine the key amino acid residues on both Shc and ERα interacting domains, which is currently conducted in my laboratory. In our previous study [3], we demonstrated that the PTB and SH2 domains of Shc are required for direct interaction with ERα—via the N-terminal activation-function-1 domain (aa 1-263) of ERα, the primary candidates for the Shc binding sites on ERα are phosphotyrosine residues (pY), which are preferential PTB and SH2 domain targets and within the first 263 amino acids of ERα. However, further investigations are required to decipher the key amino acids on these domains. We therefore will begin our work to zero in Shc binding sites on ERα by constructing vectors expressing ERα mutants. The priority of mutant selection—Y-131 for PTB and Y-150 for the SH2 has been selected based on a preliminary evaluation in which a SeqWeb search of pY-residue–containing PTB or SH2 domain recognition motifs, such as NPXpY, pYEEI and PPxY, in the N-terminal ERα protein sequence (**Fig. 2**). We will construct these mutant ERα expression vectors with single site mutation soon and further test the effect of these mutants in estrogen rapid action.

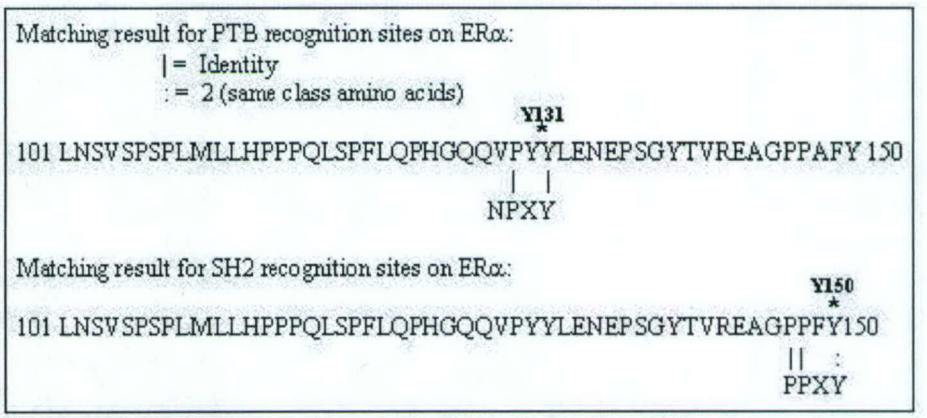

Fig. 2. The potential recognition sites of PTB and SH2 domains on ERα.

Future study:

Our initial goal is to study the mechanism of estrogen in tumor development in breast cancer cells. COS-1 cells are lack of endogenous ERα and the data generated from this model only represent the general responses to estrogen, but cannot represent the estrogen-induced biological events in human breast cancer cells. To make possible more refined studies in the field of membrane-associated ERα in breast cancer cells, we propose to employ a new technology we have used successfully within the last year and now we plan to create designer breast-cancer cell lines (MCF-7) in which the endogenous ERα has been eliminated and replaced with constructs that express forms of the ERα that are either nuclear, cytosolic, or membrane-associated. The availability of this array of knockout/knock-in cell lines will provide a powerful system for us and others to use in our efforts to discriminate the genomic and nongenomic biological functions of ERα. Currently, this work is conducting in my laboratory.

REPORTABLE OUTCOMES since this grant was awarded

A) Publications -- Since the award was funded, I have the following papers published:

- Song* RX, Zhenguo Zhang, and Richard J. Santen. Down-regulation of Bcl-2 synergistically potentiating apoptotic action of estrogen in long-term estrogen-deprived, but not in wild type MCF-7 cells. (Submitted to **J. Natl. Cancer Inst.**)
- Santen, R. J., Song, R. X., Zhang, Z., Yue, W., Kumar, R. Adaptive Hypersensitivity to Estrogen: Mechanism for Sequential Responses to Hormonal Therapy in Breast Cancer. **Clin Cancer Res** 2004, 10: 337S-345.
- Song* RX, Chris Barne, Zhenguo Zhang, Yongde Bao, Rakesh Kumar and Richard J. Santen. A novel role of Shc and IGF-1R in mediating the translocation of ERα to the plasma membrane in human breast cancer cells. **PNAS** 2004, 101(7), 2076-2081.
- Zhenguo Zhang, Rakesh Kumar, Richard J. Santen and Robert X-D. Song*. The role of adapter protein Shc in estrogen non-genomic action. **Steroids** 2004, 69(8-9), 523-529
- Santen, R.J.; Song, R.X.; Zhang,Z.; Kumar,R.; Jeng,M.H.; Masamura,S.; Yue,W.; Berstein,L. International Congress on Hormonal Steroids and Hormones and Cancer: Adaptive hypersensitivity to estrogen: mechanism for superiority of aromatase inhibitors over selective estrogen receptor modulators for breast cancer treatment and prevention. **Endocr.Relat Cancer** 2003, 10(2) 111-130.
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- Song RX, Santen RJ, Kumar R, Liana L, Jeng MH, Masamura S and Yue W. Adaptive mechanisms induced by long-term estrogen deprivation in breast cancer cells. **Molecular and Cellular Endocrinology** 2002, 193: 29-42.
- Song* RX, Berstein LM and Santen RJ. Response, Re: Effect of Long-Term Estrogen Deprivation on Apoptotic Responses of Breast Cancer Cells to 17(beta)-Estradiol. **J.Natl.Cancer Inst**. 2002; 94(15) 1173-75.

B) Presentation -- The results from this grant was presented in the following meetings:

1. Oral presentation entitled "A novel role of EGFR and Shc in ER alpha membrane translocation and activation of MAPK in breast cancer cells" in Endo 2004, in New Orleans, June 16-19
2. Invited talk in one of symposium on the interaction between the membrane estrogen receptor and growth factor receptor in breast cancer. 43rd Annual Meeting of the American Society for Cell Biology, San Francisco, California, Dec. 13-17, 2003.
3. Invited speaker in one of symposiums "Recognition of estrogen receptor in estrogen non-genomic action". Title: Involvement of Shc in estrogen receptor membrane association in breast cancer cells. FASEB – Cell Biology, San Diego, 4-14-2003.
4. Invited speaker. Title: Upstream signaling pathways in estrogen action on MAPK activation in breast cancer cells. Department of Biochemistry, University of California – Riverside, 4-16-2003.
5. Invited speaker. Title: Upstream signaling pathways in estrogen action on MAPK activation in breast cancer cells. Department of Microbiology of UVA, Charlottesville, VA, 4-8-2003.

6. Presenting in Division of Endocrinology, Department of internal medicine, UVA, Charlottesville, 6-3-2003. Title: Non-genomic signaling pathway of estrogen and breast cancer.

CONCLUSIONS:

The membrane-associated ERα is important in estrogen biological function, which requires adapter protein Shc as second messenger, leading to MAP kinase activation and cell growth stimulation.

ABBREVIATIONS:

E2	17β-estradiol, estradiol or estrogen
ERα	estrogen receptor α
ERE	estrogen response element
MAP kinase	mitogen activated protein kinase
pY	phosphotyrosine

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10. Song, R. X., Barnes, C. J., Zhang, Z., Bao, Y., Kumar, R., and Santen, R. J. The role of Shc and insulin-like growth factor 1 receptor in mediating the translocation of estrogen receptor alpha to the plasma membrane. Proc.Natl.Acad.Sci.U.S.A, *101*: 2076-2081, 2004.

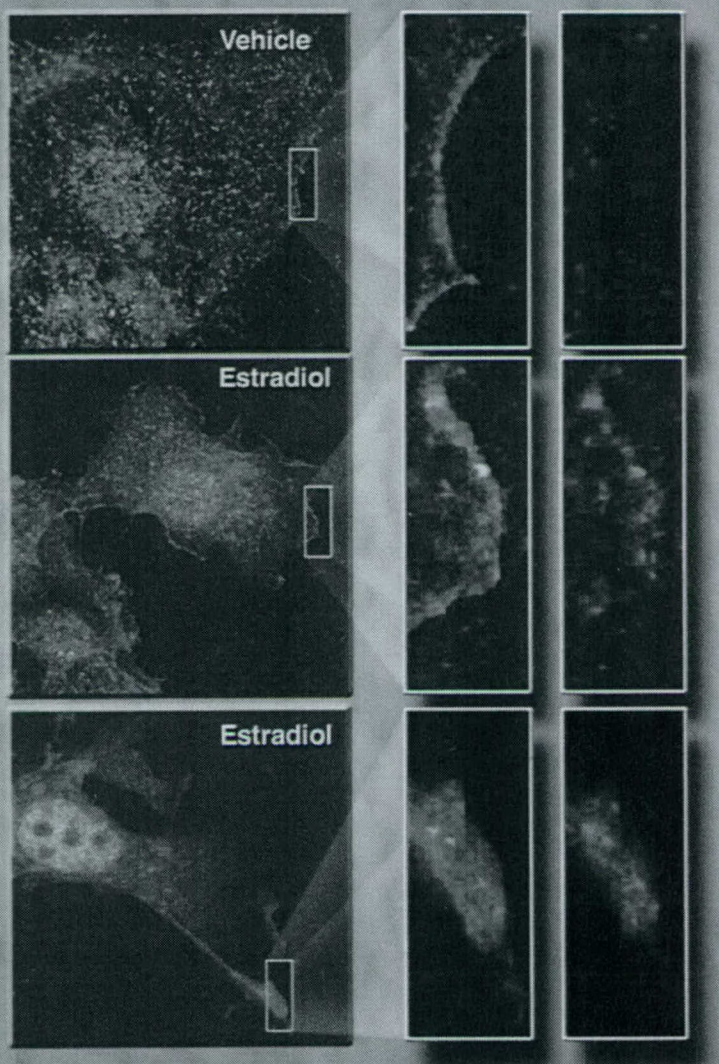
APPENDICES

1. Cover Figure for Molecular Endocrinology, 2002, 16; 116-127
2. My paper published in MOLECULAR ENDOCRINOLOGY
3. My paper published in BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
4. My paper published in PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES

MOLECULAR ENDOCRINOLOGY

Volume 16, Number 1 January 2002

Rapid Effect of Estradiol on ERα Membrane Localization and Cell Morphology



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Molecular Endocrinology 16(1):116–127

Linkage of Rapid Estrogen Action to MAPK Activation by ERα-Shc Association and Shc Pathway Activation

ROBERT X.-D. SONG, ROBERT A. MCPHERSON, LIANA ADAM, YONGDE BAO, MARGARET SHUPNIK, RAKESH KUMAR, AND RICHARD J. SANTEN

Departments of Internal Medicine (R.X.-D.S., R.A.M., M.S., R.J.S.) and Biomolecular Research Facility (Y.B.), University of Virginia School of Medicine, Charlottesville, Virginia 22908; and Department of Molecular & Cellular Oncology (L.A., R.K.), University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030

E2 rapidly activates MAPK in breast cancer cells, and the mechanism for this effect has not been fully identified. Since growth factor-induced MAPK activation involves signaling via the adapter protein Shc (Src-homology and collagen homology) and its association with membrane receptors, we hypothesized that breast cancer cells utilize similar signaling mechanisms in response to E2. In the present study, we demonstrated that E2 rapidly induced Shc phosphorylation and Shc-Grb2 (growth factor receptor binding protein 2)-Sos (son of sevenless) complex formation in MCF-7 cells. Overexpression of dominant negative Shc blocked the effect of E2 on MAPK, indicating a critical role of Shc in E2 action. Using selective inhibitors, we also demonstrated that ERα and Src are upstream regulators of Shc. A rapid physical association between ERα and Shc upon E2 stimulation further evidenced the role of ERα on Shc activation. Mutagenesis studies showed that the phosphotyrosine binding and SH2 domains of Shc are required to interact with the activation function 1, but not activation function 2, domain of ERα. Using a glutathione-*S*-transferanse-Shc pull-down assay, we demonstrated that this ERα-Shc association was direct. Biological consequences of this pathway were further investigated at the genomic and nongenomic levels. E2 stimulated MAPK-mediated Elk-1 transcriptional activity. Confocal microscopy studies showed that E2 rapidly induced formation of membrane ruffles, pseudopodia, and ERα membrane translocation. The E2-induced morphological changes were prevented by antiestrogen. Together our results demonstrate that ERα can mediate the rapid effects of E2 on Shc, MAPK, Elk-1, and morphological changes in breast cancer cells (*Molecular Endocrinology* 16: 116–127, 2002)

E2 IS a steroid hormone that plays an important role in the genesis of human breast cancer and in the growth of established tumors (1). Classically, E2 elicits genomic effects on transcription via ERα and ERβ, which are mainly located in the nucleus. As ligand-dependent transcriptional factors, the hormone-bound ERs interact with estrogen response elements to stimulate certain genes in E2-responsive tissues and to regulate gene transactivation (2). The genomic actions of E2 are characterized by delayed onset of action and sensitivity to inhibitors of transcription and translation. Many of the E2-induced transcriptional mechanisms involve ERα, which can be functionally regulated by posttranslational phosphorylation of serine residues at the 104, 106, 118, 167, and 236 positions (3–6). In addition, tyrosine-537 of ERα has been reported to be phosphorylated in MCF-7 cells and in ERα-expressing Sf9 insect cells (7). The biological function of ERα is also regulated by association with a subset of nuclear proteins, such as coactivators, corepressors, and integrator proteins (8–11).

More recently, investigators have recognized rapid nongenomic actions of E2 on several cellular processes, such as activation of Ras (12), Raf-1 (13), PKC (14), PKA (15), Maxi-K channels (16), increments in intracellular calcium levels (17), and an increase of nitric oxide (17). Elicited responses depend upon the cell types studied and the conditions used. In contrast to its transcriptional effects, the precise nongenomic signaling pathways of E2, especially the involvement of ERα in this process, are not well understood and have not been extensively studied (18–20).

Several recent reports demonstrated that E2 rapidly activates MAPK in a number of model systems (13, 21, 22), but the mechanisms responsible for this are still controversial (23–26). Many growth factor receptors on the cell membrane, such as the receptors for epidermal growth factor (27), nerve growth factor (28), platelet-derived growth factor (27), and IGF (29), activate MAPK through a Shc-mediated pathway. We hy-

Abbreviations: AF-1 and AF-2 domains, Activation function 1 and 2 domains; CH domain, collagen homology domain; EGFR, epidermal growth factor receptor; Grb2, growth factor receptor binding protein 2; GST, glutathione-*S*-transferase; HEGO, human ERα expression vector; ICI, ICI 182 780; IMEM, improved MEM; LTED cells, MCF-7 cells growing long-term in estrogen-depleted medium; MEK, MAPK kinase; PTB domain, phosphotyrosine binding domain; PVDF, polyvinylidene difluoride; Shc, Src-homology and collagen homology; ShcWT, wild-type Shc; Sos, Son of Sevenless; Src, Src family of tyrosine kinases; c-Src, p60 of Src.

pothesized that E2 might co-opt a pathway using Shc-Grb2-Sos (Src homology-growth factor receptor binding protein 2-son of sevenless) to activate MAPK. The adapter protein Shc has no intrinsic kinase domain and transduces signals dependent on the association with membrane receptors. Three domains mediating protein-protein interactions have been reported on Shc. Two of these, the phosphotyrosine binding (PTB) domain in the amino-terminal region and the Src homology 2 (SH2) domain in the carboxy-terminal region are separated by a region rich in proline and glycine residues, called the collagen homology (CH) domain (30). Shc binds rapidly to the specific phosphotyrosine residues of receptors through its PTB or SH2 domain and becomes phosphorylated itself on tyrosine residues of the CH domain (27). The phosphorylated tyrosine residues on the CH domain provide the docking sites for the binding of the SH2 domain of Grb2 and hence recruit Sos, a guanine nucleotide exchange protein. This adapter complex formation allows Ras activation via the Sos protein, leading to the activation of the MAPK pathway (27, 31, 32). Since ERα has been reported to be associated with the membrane of MCF-7 cells (20), we hypothesized that this receptor might be involved in mediating E2 action on MAPK activation by physical interaction with Shc in a manner analogous to that in growth factor pathways.

To test our hypothesis, we used MCF-7 and its variant LTED cells. LTED cells were developed from parental MCF-7 cells growing long-term in estrogen-depleted medium (33). This maneuver causes a 5- to 10-fold up-regulation of ERα expression (33). In the present study, we demonstrated that E2 rapidly stimulated both MAPK and Shc phosphorylation and induced the association of Shc with ERα. Using selective inhibitors and a dominant mutant Shc, we further demonstrated that Shc is upstream of MAPK and that the rapid actions of E2 are ERα-dependent and involve ERα/Shc association. Furthermore, a direct physical association of ERα with Shc was evidenced by protein pull-down assay. Our results show that the PTB and SH2 domains of Shc interacted with the AF-1 region of ERα. Tyrosine-537 of ERα was not involved in this interaction. Measurement of Elk-1 activation and morphological changes by confocal microscopy provided further evidence of the rapid, nongenomic role of E2 in these cells. Together, our data suggest that the nongenomic effects of E2 on Shc and MAPK activation are mediated by the classical ERα receptor, that E2 can induce an association of ERα with Shc, and that Shc appears to be a crucial step for the activation of MAPK.

RESULTS

Effects of Selective Inhibitors and Dominant-Negative Shc on E2-Induced MAPK Phosphorylation

Prior studies reported that E2 rapidly activated MAPK in MCF-7 cells (34). To investigate the upstream signals mediating E2 action on MAPK, we first confirmed that E2 increased MAPK activation in our MCF-7 cells by measuring MAPK phosphorylation. Figure 1A shows that E2 increased both p42 and p44 MAPK phosphorylation in a time-dependent manner with the peak at 15-min treatment. We then questioned

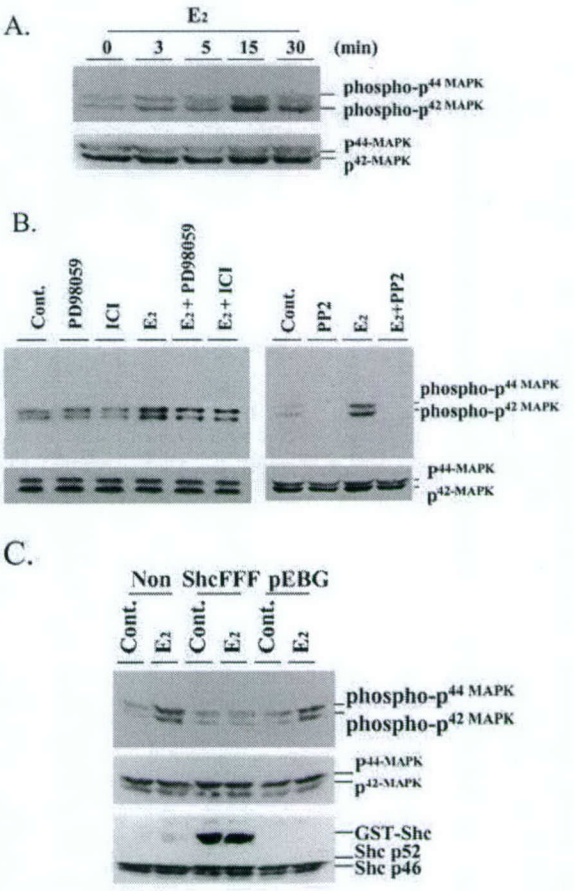

Fig. 1. MAPK Phosphorylation in MCF-7 Cells

A, E2-induced MAPK phosphorylation. MCF-7 cells were treated with vehicle or E2 at 10^{-10} M for the times indicated. The phosphorylation status of MAPK (p42 and p44) from whole-cell lysates was assessed using an antibody recognizing dual phosphorylated MAPK (*top panel*). Levels of total MAPK protein were determined on the same blot to control for loading variations (*bottom panel*). The positions of phosphorylated and total MAPK proteins are indicated at the *right*. B, Effects of pathway-selective inhibitors on E2-induced MAPK phosphorylation. MCF-7 cells were pretreated with 23 μM PD98059, 10^{-9} M ICI 182 780 (ICI), and 5 nM PP2 for 30 min. Cells were then treated with vehicle or 10^{-10} M E2 for 15 min. The phosphorylated (*top panel*) and total MAPK (*bottom panel*) were determined. C, Overexpression of mutant Shc blocked E2-induced MAPK phosphorylation. MCF-7 cells were either not transfected or transfected with the mutant Shc (ShcFFF) or control vectors (pEBG) for 2 d. Then cells were treated with vehicle or 10^{-10} M E2 for 15 min. The phosphorylated (*top panel*) and total MAPK (*middle panel*) were detected. Both GST-ShcFFF and endogenous Shc expression are shown (*bottom panel*). The above experiments have been repeated three times, and one of the representative experiments is shown.

whether the effects of E2 on MAPK involved ERα, MEK (MAPK kinase), and Src tyrosine kinase. As shown in Fig. 1B, the antiestrogen ICI, the MEK inhibitor PD98059, and a pan Src inhibitor PP2 all blocked E2-induced MAPK phosphorylation, implying that ERα, MEK, and Src family members are required for this step. PP2 alone abolished basal MAPK phosphorylation, suggesting the role of Src in maintaining basal MAPK function (Fig. 1B).

Activation of MAPK through classical growth factor signaling pathways involves the phosphorylation and activation of the adapter protein Shc. To test whether Shc is necessary for E2-induced MAPK phosphorylation, we transiently transfected MCF-7 cells with a dominant-negative Shc mutant. The mutant is a glutathione-*S*-transferase (GST) fusion protein with phenylalanines substituted for tyrosines at the 239/240 and 317 positions of the Shc CH domain (ShcFFF), rendering it defective in signaling to MAPK (35, 36). Expression of ShcFFF decreased E2-induced MAPK phosphorylation compared with untransfected and empty vector (pEBG)-transfected cells (Fig. 1C). Together, these results indicated that ERα, Src, and Shc are all upstream components regulating MAPK activation.

E2 Rapidly Induces Shc Pathway Activation

To further confirm the involvement of Shc in the E2 rapid signaling pathway, we evaluated this step in both MCF-7 and LTED cells that express a 10-fold elevation of ERα levels. As shown in the *top panel* of Fig. 2A, E2 at 10^{-10} M increased p52 kDa Shc phosphorylation in a time-dependent manner with the maximum response at 3 min. When LTED cells were compared with MCF-7 cells, Shc was phosphorylated to a greater extent in LTED cells (*left* in Fig. 2A) than parental MCF-7 cells (*right* in Fig. 2A) under basal conditions and increased proportionately in response to E2. To validate the results, the polyvinylidene difluoride (PVDF) membranes were reprobed with anti-Shc antibodies. The results show a similar amount of Shc proteins of both p46 and p52 kDa in MCF-7 and LTED cells (Fig. 2A, *bottom panels*). E2-induced Shc phosphorylation was also dose-dependent with the optimal dose at 10^{-10} M (data not shown).

Classical pathways of Shc activation by growth factors involve Shc association with the adapter proteins, Grb2 and Sos. Accordingly, we examined the effects of E2 on Shc-Grb2-Sos complex formation. LTED cells were used in this study because of their enhanced Shc phosphorylation in response to E2. As shown in Fig. 2B, Grb2/Shc (*left panel*) and Grb2/Sos (*middle* and *right panels*) complexes were faintly apparent in the vehicle-treated group, but greatly increased by 10^{-10} M E2 treatment at 3 min. Together, these results suggest that E2 is capable of activating the Shc-mediated signaling pathway in MCF-7 cells, which is regulated by the level of ERα expression.

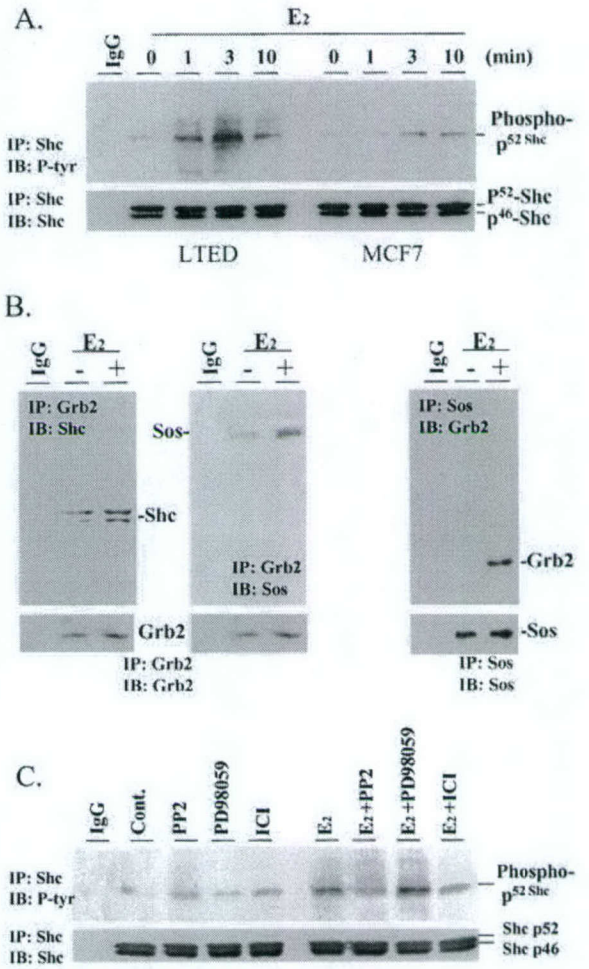

Fig. 2. E2-Induced Shc Pathway Activation in MCF-7 and Its Variant Cells

A, Effects of E2 on Shc phosphorylation. Both LTED and MCF-7 cells were treated with vehicle or 10^{-10} M E2 for the times indicated and then extracted as described in *Materials and Methods*. Shc phosphorylation was measured by immunoprecipitation (IP) of Shc protein and detection of tyrosine phosphorylation (*top panel*) on immunoblot (IB). To normalize Shc protein loading, the membranes were stripped and reprobed with polyclonal anti-Shc antibodies (*bottom panel*). Nonspecific monoclonal or polyclonal IgG were employed in all immunoprecipitation (IP) steps of the following experiments as negative controls. B, Effects of E2 on Shc-mediated adapter protein complex formation. LTED cells were treated with vehicle or E2 at 10^{-10} M for 3 min. The association of Grb2/Shc and Grb2/Sos was measured by immunoprecipitation of Grb2 and detection of Shc (*left top panel*) or Sos (*middle top panel*) on Western blots. The same membrane was reprobed with antibodies against Grb2 to detect Grb2 protein loading (*left* and *middle bottom panels*). The Grb2/Sos association was further confirmed by reciprocal antibody method (*right panel*). C, Effects of the selective inhibitors on E2-induced Shc phosphorylation in MCF-7 cells. MCF-7 cells were pretreated with 5 nM PP2, 23 μM PD98059, and 10^{-9} M ICI for 30 min. Cells then were challenged with vehicle or 10^{-10} M E2 for 3 min. The phosphorylation of Shc was determined as described in *Materials and Methods* (*top panel*). The membrane was reprobed with anti-Shc antibodies to show Shc protein loading (*bottom panel*). Above experiments were done three times and a representative experiment is shown.

Effects of Selective Signaling Pathway Inhibitors on E2-Induced Shc Phosphorylation

Because Shc is responsible for the activation of MAPK by E2, we wished to determine components responsible for Shc phosphorylation. Additionally, we wished to demonstrate a role for ERα in this process and to exclude MAPK as the cause of Shc phosphorylation. Shc has been reported to be a substrate of c-Src in HEK-293 cells (37). Accordingly, we examined the effects of PP2, ICI, and PD98059 on E2-induced Shc phosphorylation. In the presence of the inhibitors, MCF-7 cells were stimulated with vehicle or 10^{-10} M E2 for 3 min, and the status of Shc phosphorylation was examined. Figure 2C shows that both PP2 and ICI effectively inhibited E2-induced Shc phosphorylation, indicating that Src family kinases and ERα are required for Shc activation. As expected, PD98059 did not influence Shc phosphorylation status, suggesting that MAPK functions downstream of Shc. No effects of these inhibitors were apparent in the absence of E2 stimulation. These results indicate that both ERα and Src are upstream components of Shc functionality, and their involvement is required for Shc phosphorylation.

E2-Stimulated ERα Association with Shc

We postulated that ERα might mediate effects of E2 on Shc by directly or indirectly associating with the Shc adapter protein. This concept was based on the fact that activated Shc transduces signals by associating with membrane receptors and our evidence that ICI blocked E2-induced Shc phosphorylation in MCF-7 cells. To test this hypothesis, we immunoprecipitated Shc protein and detected ERα on Western blots. As shown in Fig. 3, E2 rapidly induced the association of ERα with Shc in both LTED and MCF-7 cells (*top panel*). The induction was seen as early as 1 min of E2 treatment and reached a plateau from 3 to 10 min. Compared with MCF-7 cells, ERα/Shc complexes were present basally in LTED cells, which express 5- to 10-fold higher levels of ERα, and greatly increased in response to E2 treatment. Consistency of protein loading was confirmed by reprobing the membrane with anti-Shc antibody (*bottom panel*). Thus, the association between ERα and Shc was observed not only in MCF-7 cells, but it was greatly enhanced in ERα up-regulated LTED cells. The results were also confirmed by the immunoprecipitation of ERα and the detection of Shc on immunoblot (data not shown). Together, our data suggest that as an upstream signal of Shc, the level of ERα expression affects the level of Shc phosphorylation and ERα/Shc association.

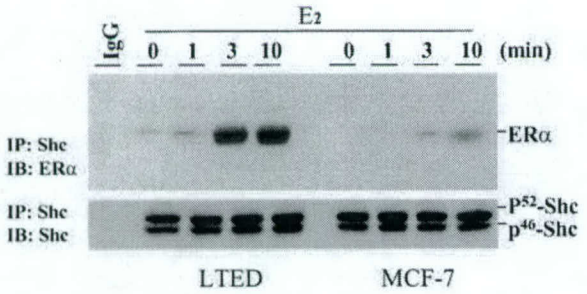

Fig. 3. E2 Rapidly Induced the Physical Association Between ERα and Shc in LTED and MCF-7 Cells

Cells were treated with vehicle or 10^{-10} M E2 for the times indicated. Subsequently, the association of ERα with Shc proteins was determined by the immunoprecipitation of Shc and detection of coimmunoprecipitated ERα protein on immunoblot (*top panel*). The same membrane was stripped and reprobed with anti-Shc antibodies to normalize the Shc protein loading (*bottom panel*). The positions of ERα and Shc proteins are indicated at the *right*. The experiment has been done three times, and one of the representative experiments is shown.

Study of Molecular Basis of ERα/Shc Interactions

To study the molecular basis of the interacting domains on ERα and Shc, various GST-tagged Shc and ERα mutants were employed (Fig. 4A). Furthermore, to minimize factors specific to breast cancer cells, COS-1 cells that do not have detectable endogenous ERα were used and electroporated with different ERα and Shc expressing vectors (Fig. 4A). First, we examined the Shc-interacting domain(s) on ERα by electroporating wild-type Shc (ShcWT) with either wild-type ERα expressing vector (HEGO) or a mutant ERα expressing vector in which phenylalanine was substituted for tyrosine in the 537-position (Y537F). As shown in Fig. 4B, the Y537F mutation did not alter the Shc/ERα complex formation compared with wild-type ERα, suggesting that Y537 of ERα is not the site interacting with Shc. Coexpression of either the truncated activation function 1 (AF-1) or activation function 2 (AF-2) domains of ERα with ShcWT in COS-1 cells confirmed that it is the AF-1, but not AF-2 (contains Y537), domain of ERα that interacts with Shc (Fig. 4, C and D). E2 increased the association of AF-1 with Shc (Fig. 4C). The mechanism for this is not known and was unexpected, since AF-1 does not contain a binding site for E2. We postulate that E2 might exert effects on AF-1 or Shc binding proteins independently of ERα, leading to an increase of AF-1/Shc association.

Next we further mapped the ERα-interacting site(s) on Shc. When ShcWT was coexpressed with ERα (HEGO) in COS-1 cells, we demonstrated an association of GST-Shc protein with ERα under basal conditions and further enhancement of this association with 5 min of E2 treatment (Fig. 4E, *top left panel*). We then used selective domain-expressing Shc mutants to examine which domains of Shc were necessary for this association. Both the PTB (ShcPTB)- and the SH2 (ShcSH2) domain-expressing proteins formed complexes with ERα, which further increased in intensity upon E2 stimulation (Fig. 4E, *top right panel*). Proteins that only expressed the GST or CH domain of Shc failed to interact with ERα. After normalizing the GST-Shc immunoprotein bands (Fig. 4E, *top panels*) for

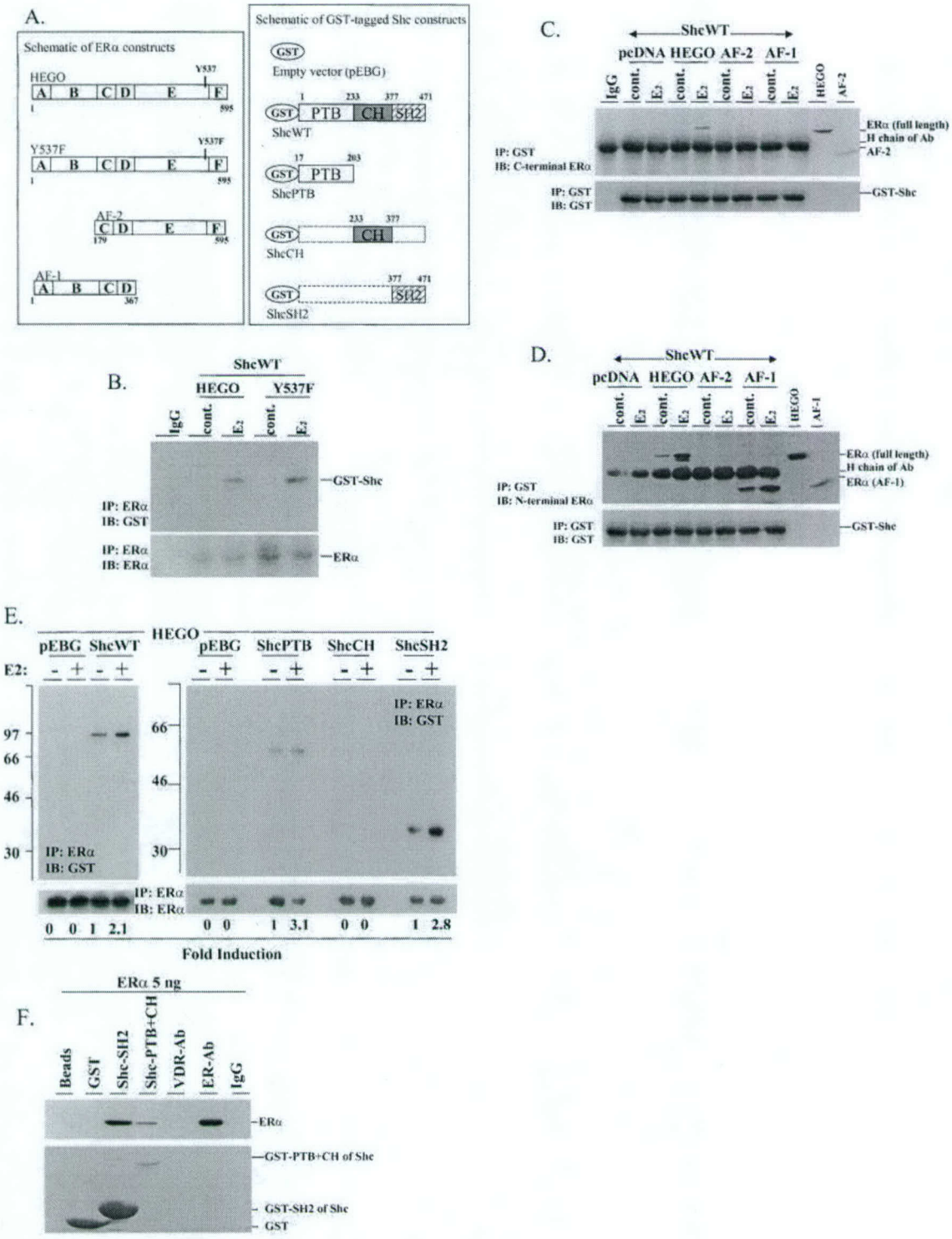

Fig. 4. Study of Shc/ERα Interaction

A, Schematic ERα and GST-tagged Shc constructs for eukaryotic expression. In *left panel*, HEGO is wild-type ERα-expressing vector. Y537F is a vector expressing mutant ERα in which the tyrosine-537 was mutated into phenylalanine. The AF-1 vector encodes amino acids 1–367 of ERα and AF-2 encodes 179–595. In the *right panel*, the GST-Shc fusion protein-expressing vectors are shown. The *oval region* in the N terminus of Shc corresponds to the GST fusion protein. The *white, gray*, and *diagonally lined rectangles* represent the PTB, CH, and SH2 domains of Shc, respectively. B, Examination of tyrosine-537 of ERα interaction with Shc. Wild-type Shc vector (ShcWT) was cotransfected with either HEGO or Y537F ERα-expressing vectors into COS-1 cells. Cells were then treated with vehicle or 10^{-10} M E2 for 5 min, and the ERα/Shc association was assessed. C and D, The AF-1 domain of ERα is required to interact with Shc. COS-1 cells were cotransfected with GST-tagged wild-type Shc (ShcWT) and different ERα constructs as indicated for 3 d. Cells were then treated with vehicle or 10^{-10} M E2 for 5 min. The ERα-Shc

ERα protein loading (Fig. 4E, *bottom panels*), we show that the Shc/ERα association induced by E2 is 2- to 3-fold higher than the control in cells expressing PTB or SH2 and is similar to that in cells containing ShcWT.

Shc can be a substrate of c-Src that associates with ERα in MCF-7 cells after E2 treatment (38). It would be of interest to know whether c-Src is involved in this ERα-Shc complex in our breast cancer system. Using several different anti-c-Src (B-12, N-16, and SRC2, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-ERα antibodies (D-12 and MC-20, Santa Cruz Biotechnology, Inc.), we did not detect c-Src in ERα-Shc complexes of either MCF-7 or LTED cells (data not shown). To further address the molecular basis of Shc-ERα interaction, we tested whether this interaction is direct or mediated by third-party proteins using GST-pull-down assays. Both GST-tagged SH2 and PTH+CH domain fusion proteins of Shc were produced at the size of 37 kDa and 68 kDa (Fig. 4F, *bottom panel*). The 62-kDa recombinant full-length ERα was pulled down by both GST-tagged Shc proteins (Fig. 4F, *top panel*). As negative control, the beads and GST protein alone did not pull down the ERα protein. The specificity was confirmed by immunoprecipitation and detection of ERα with anti-ERα antibodies, but not by the nonspecific or anti-vitamin D receptor antibodies. Together, our results demonstrate that the interaction between ERα and Shc is direct and does not require the presence of any other proteins.

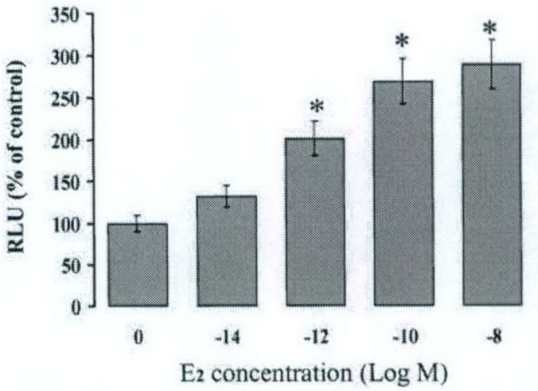

Fig. 5. E2-Stimulated MAPK Substrate Elk-1 Activation

A transient transfection method was used to study MAPK-mediated Elk-1 activation. MCF-7 cells were cotransfected with GAL4-Elk-1 and GAL4-E1B-Luciferase vectors. Twenty-four hours after transfection, cells were stimulated with vehicle or different doses of E2 for 6 h. Luciferase activity for each lysate was determined. The data are presented as mean ± SD of three experiments. *, $P < 0.05$ compared with untreated control.

Biological Effects of E2-Induced Nongenomic Pathway Activation

To further provide evidence that the ERα-Shc-MAPK pathway is biologically relevant, we used two approaches. First, we evaluated the role of MAPK on the activation of Elk-1, which is a transcriptional factor phosphorylated and activated by MAPK (39). Second, we examined the rapid effects of E2 on morphological changes of MCF-7 cells.

To study Elk-1 activation by E2, a well characterized reporter system utilizing both GAL4-Elk-1 and GAL4-luciferase vectors (40) was cotransfected into MCF-7 cells. Figure 5 shows that E2 increased Elk-1 activation in a dose-dependent manner at 6 h as shown by luciferase assay. The E2 doses higher than 10^{-12} M significantly stimulated Elk-1 activation, indicating the involvement of MAPK in E2 action.

To further provide direct biological evidence of E2 stimulation of Shc-MAPK pathways, effects of E2 on cell morphology were examined. We reasoned that E2 might elicit effects similar to those induced by exogenous growth factors since it used a pathway normally activated by growth factors, namely the Shc-MAPK pathway. Our coauthors recently used the confocal microscopic method to demonstrate that heregulin induces several rapid cellular effects, such as formation of membrane ruffles, filopodia, pseudopodia, actin polymerization, and loss of focal adhesion kinase plaques (41). Using identical methods, we studied rapid cell morphological changes and their substructures using LTED cells due to their strong responses

association (*top panels*) was measured by immunoprecipitation of GST and detection of ERα on immunoblot using either anti-C (panel C) or anti-N-terminal (panel D) ERα antibodies. The membrane was reprobed with anti-GST antibodies to normalize the protein loading (*bottom panels*). To confirm the positions of AF-1 and AF-2, the ER-transfected COS-1 cell extracts were loaded on the same gels, and the expression of immunoprotein bands of HEGO, AF-1, and AF-2 is shown on the *right sides* of the *top panels*. E, Mapping the ERα-interacting domains on Shc. Wild-type ERα (HEGO) was transiently transfected into COS-1 cells with different GST-tagged Shc expression vectors as indicated. On d 3, cells were extracted, and the interactions between ERα and wild-type Shc (*top left panel*) or mutated Shc proteins (*top right panel*) were measured as described in *Materials and Methods*. The same membranes were stripped and reprobed with anti-ERα antibody to normalize the protein loading (*bottom panels*). Quantitation of the GST-Shc immunoprotein bands normalized by ERα protein loading is shown as fold induction. All figures are representative blots of the study that was performed three times. F, GST pull-down assay of ERα-Shc interaction. GST-tagged fusion proteins expressing Shc-selective domains were purified by the beads of Glutathione Sepharose 4B. Beads (15 μl) conjugated with or without GST, GST-SH2, or GST-PTB+CH domains of Shc were incubated with 5 ng recombinant full-length human ERα protein overnight. To control the specificity, the full-length human ERα was also incubated with anti-vitamin D receptor, anti-ERα, or nonspecific immunoglobulin (IgG) as indicated. After washing the beads three times, the eluted proteins were resolved on 10% SDS-PAGE, transferred onto a PVDF membrane, and detected with anti-ERα antibody (*top panel*). The gel was stained with Coomassie blue to show the positions of the Shc fusion proteins (*bottom panel*). One of four experiments is shown.

with respect to ERα/Shc association and Shc phosphorylation upon E2 treatment.

Under basal conditions (Fig. 6A), LTED cells exhibited a low level of membrane ruffles (*red*), indicating minimal actin polymerization sites. ERα (*green*) appeared to be predominantly in the nucleus with some also present in the cytoplasm. Beginning at 10 min (data not shown) and increasing by 20 min, E2 induced dramatic morphological effects, including the formation of additional membrane ruffles and cell shape alterations (Fig. 6B). Even more dramatic was the formation of pseudopodia shown in Fig. 6C as an arm-like extension of the cell membrane with a fist-like structure at its terminus. To demonstrate that ERα mediated these morphological responses, the effects of E2 were abrogated by coadministration of the pure antiestrogen ICI along with E2 (Fig. 6D). ICI alone resulted in no morphological changes (Fig. 6E).

Use of confocal microscopy and immunofluorescence provided a dynamic means of assessing ERα location and alterations in response to E2. Accordingly, we focused on the region contiguous to the cell membrane. The *insets* in Fig. 6F show enlarged areas indicated by *arrows* in panels A–E. Under basal conditions, minimal *green* staining could be observed along the cell membrane of the cells (Fig. 6A, *inset a, right panel*). In marked contrast, E2 appeared to translocate ERα into the region along the membrane ruffles as indicated by the strong appearance of *green* staining (Fig. 6B, *inset b,right panel*). As shown by merging the *red* (actin) and *green* (ERα) views, the ERα appeared as *yellow* (Fig. 6B, *inset b, left panel*), indicating colocalization with actin in the membrane ruffles. Striking also was the translocation of the ERα into the fist-like region of the pseudopodia as shown by both the *green* staining (Fig. 6C, *inset c, right panel*) and *yellow* merged views (Fig. 6C, *inset c, left panel*). ICI blocked E2-induced ERα membrane translocation with little effect by it alone (Fig. 6, D and E; *inset d, right* and *left panels*; and *inset e, right* and *left panels*). The immunofluorescence staining

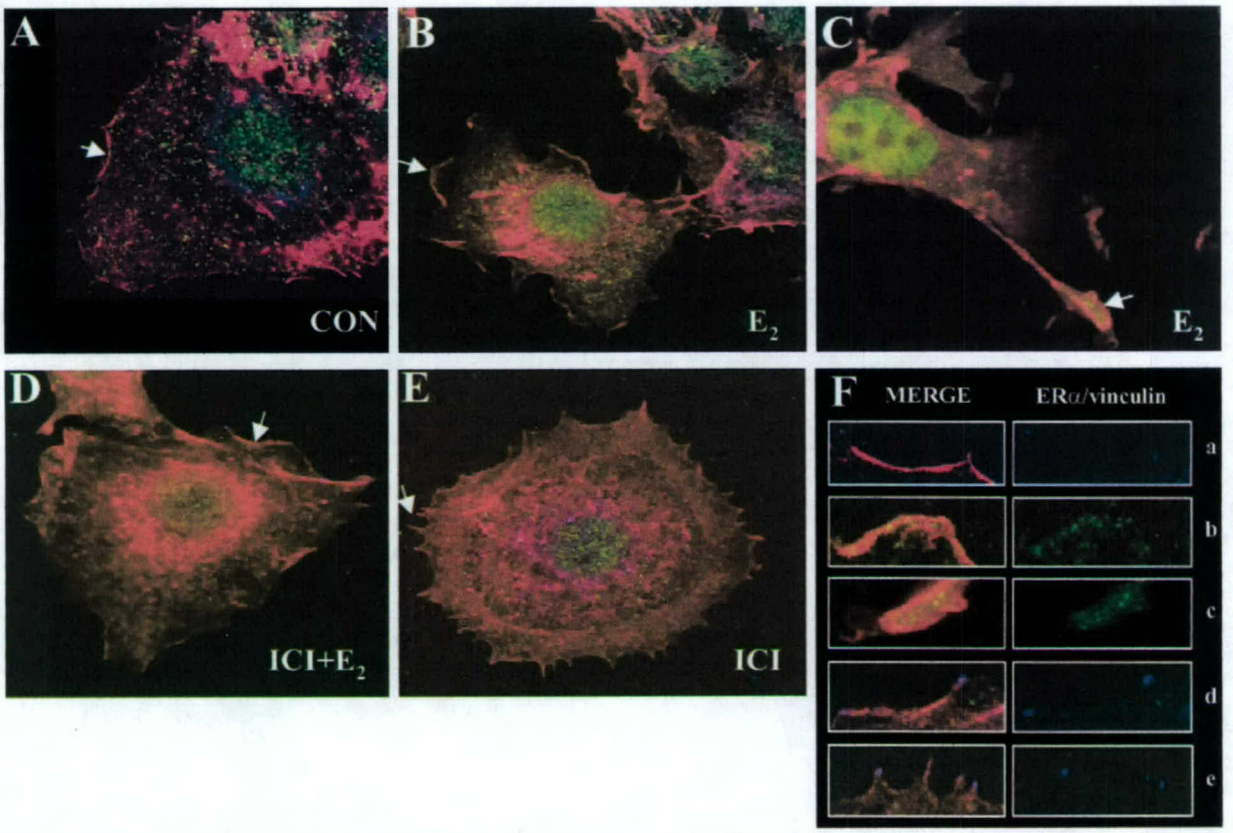

Fig. 6. Confocal Analysis of E2-Induced Morphological Changes and ERα Subcellular Localization in LTED Cells

Three-color-merged images are shown in panels A–E. *Red fluorescent color* indicates filamentous actin; *green* represents ERα; and *blue* indicates vinculin. Panel A illustrates cells grown under control conditions without E2. The *arrow* is directed toward a membrane ruffle that consists of concentrated bundles of filamentous actin. The regions indicated by the *arrow* in this and other panels (*i.e.* A–E) are further illustrated in the higher power views of specific areas shown as *insets* in panel F. Panel B illustrates cells treated with 10^{-10} M E2 for 20 min and shows the formation of additional membrane ruffles; panel C shows the formation of a pseudopodia under the same conditions. Panel D demonstrates that ICI at 10^{-9} M reduced the E2-induced ruffle formation, and panel E shows that ICI exerted no effect by itself. The *insets* in F represent the higher power views of specific areas of the membrane regions shown by *arrows* in panels A–E. On the *left panels* of the *insets* are shown the three-color ERα/filamentous actin/vinculin merged views; on the *right*, the two-color ERα/vinculin merged views are shown.

for vinculin (*blue*) represents the focal adhesion contacts of the cells that are not highly visible.

The morphological changes demonstrated in Fig. 6 represent examples of consistent changes observed in many cells. To establish that these changes were statistically significant, we used an integrated measure of membrane changes called dynamic membrane formation (DM). This integrated parameter includes both membrane ruffles and pseudopodia. As shown in Fig. 7, the E2-induced increase in DM was statistically significant and could be blocked by ICI. Collectively, these results demonstrated that E2 rapidly induced specific changes on cell morphology in addition to its effects on membrane-mediated Shc-MAPK activation.

DISCUSSION

Our studies demonstrated that MCF-7 breast cancer cells utilize a classical growth factor signaling pathway to mediate the nongenomic effects of E2. This pathway involves the direct association of ERα with Shc, the phosphorylation of Shc, and the formation of Shc-Grb2-Sos complexes. Shc is critical for E2-induced MAPK activation. Using a mutagenesis approach, we demonstrated that the SH2 and PTB domains of Shc are the sites interacting with ERα and that the AF-1 region of ERα is required to interact with Shc.

Evidence of the biological relevance of this pathway is inferred from the demonstration that Elk-1, one of the mediators of MAPK, was increased by E2. More importantly, we obtained direct biological evidence of the nongenomic effects of E2 by demonstrating morphological changes with confocal microscopy. These studies showed that E2 rapidly induced formation of membrane ruffles and pseudopodia, but also translocation of ERα to the membrane and into perimembrane regions of pseudopodia. Finally our results demonstrated that E2, mediated by ERα, activated a classical growth factor-signaling pathway involving Shc adapter proteins.

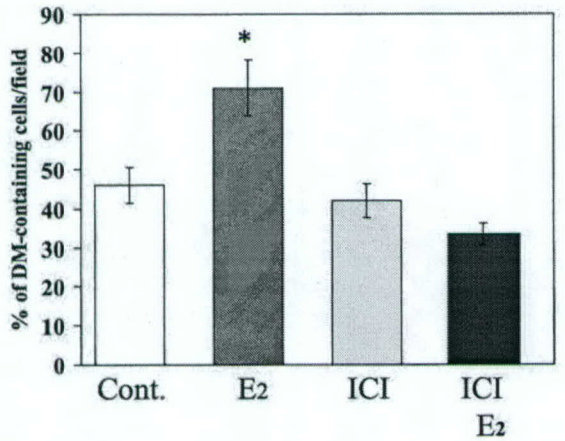

Fig. 7. Summary of Dynamic Membrane Formation in LTED Cells

The changes in ruffles and pseudopodias are combined under the term, dynamic membrane (DM), formation. Quantitative results from 12 individual cells are summarized. *, $P < 0.05$.

The adapter protein Shc mediates the effects of many growth factors to activate MAPK. However, several recent studies demonstrated different mechanisms whereby E2 can induce MAPK activation. McDonnell and associates (34) showed that calcium is required for E2-induced MAPK activation in MCF-7 cells, and this event is ERα dependent. E2-induced MAPK activation was also reported to be independent of ERα or ERβ but required the existence of epidermal growth factor receptor (EGFR) and GRP30, a G protein-coupled receptor homolog in an ERα-negative breast cancer cell line MDA-MB-231 (25). EGFR expression in MCF-7 cells is very low, and Parsons and colleagues (42) have reported that EGFR protein is not detectable in MCF-7 cells either by immunoprecipitation or by immunoblotting methods. In our system, we do not detect EGFR in either MCF-7 or its variant cells (data not shown).

In the present study, we demonstrated that ERα, as a key mediator, is required for E2 action on Shc/ERα association and Shc and MAPK phosphorylation. Approximately 0.5% of total ERα was estimated to be associated with Shc after E2 stimulation as shown by an ERα-Shc coimmunoprecipitation study (data not shown). The activation of MAPK with E2 is less than that by growth factors, as reported by other investigators (34). Our unpublished data also show that MAPK phosphorylation induced by 10 ng/ml TGFα was 35-fold higher than untreated controls, compared with 2.5-fold induction induced by 10^{-10} M E2. ERα-dependent MAPK activation has been reported by many laboratories using MCF-7 and other cell lines treated with E2 (39, 43). The differences regarding ERα involvement in MAPK activation under varying conditions are unknown. Nevertheless, it is not surprising that E2 can activate multiple rapid signaling pathways in different cell lines due to the fact that it can exert nongenomic effects on nitrous oxide, PKC, PKA, and MAPK. Detailed examination of E2 induction through each of these mechanisms will be required before a full understanding of the biological relevance of these various pathways emerges.

A single time point of Shc phosphorylation induced by E2 was first reported in MCF-7 cells (13). We extended these observations by conducting detailed time studies and showed the effects as early as 1 min after E2 treatment. ERα is required for Shc phosphorylation, and the association of ERα with Shc appears to be important for Shc activation. It has been suggested that PTB and SH2 are two distinct binding domains, which may link to phosphorylated tyrosine residues in a different manner (44). Both PTB and SH2 domains of Shc are required to associate with EGFR (45), but Neuregulin-activated ErbB2 and ErbB3 bind only to PTB domain of Shc (46). The functional roles of

the PTB or SH2 sites in their interaction with ERα are now under investigation.

The PTB and SH2 domains of Shc interact with phosphorylated tyrosine residue(s) of other proteins. The exact tyrosine residue of the ERα responsible for binding to Shc is unknown. Since tyrosine-537 of human ERα may serve as a constitutively phosphorylated site (7), we considered it a possible docking site for Shc. However, our data show that the AF-1 domain of ERα, but not the AF-2 or tyrosine-537 region, interacts with Shc, indicating that there might be other tyrosine residues phosphorylated on the N-terminal ERα. We postulated that 1 of 10 potential tyrosine residues in the AF-1 region (amino acids 1–179) might be involved in the association with Shc, such as tyrosine 43, 52, 54, 60, 73, 80, 130/131, 139, and 150. Further investigation of these sites is now being conducted in our laboratory.

ERα has been reported to associate functionally with many cell membrane proteins, such as IRS-1, the p85 subunit of PI3K, and caveolin (47–49). c-Src has been reported to physically associate with ERα of bovine uterus, but there is no direct evidence that ERα-associated c-Src is responsible for Shc phosphorylation (13). The present study demonstrated that the Src inhibitor PP2 blocked Shc phosphorylation induced by E2. This evidence suggests that a Src protein family member is functionally involved in E2-induced Shc phosphorylation, even though Src is not detectable in the ERα-Shc complex. It is possible that other Src family members are involved in phosphorylating Shc or that the Src-ERα complex is unstable during immunoprecipitation.

The breast cancer models used in our studies involve parental MCF-7 and its variant LTED cells. When compared with its parental cells, LTED cells show an increased growth rate in E2-deprived media and respond to very low concentrations of E2 when grown in nude mice (50). ERα, but not ERβ, is detectable in our MCF-7 and its variant cells (33, 50). The presence of increased amounts of ERα appears to amplify the components examined in the ERα-Shc signaling pathway. Whenever effects, such as ERα/Shc association and Shc phosphorylation, were compared in LTED and MCF-7 cells, the responses were qualitatively similar but quantitatively greater in the variants. For this reason, we used this variant for demonstrating certain effects such as Grb2/Sos interactions and morphological changes by confocal microscopy.

Our studies demonstrated rapid, nongenomic effects of E2 by two separate methods, biochemical assessment of the Shc-MAPK pathway and morphological effects on cell shape, membrane ruffles, pseudopodia, and ERα translocation. Both effects could be blocked by the antiestrogen ICI, indicating that Shc-MAPK activation and morphological changes were all initiated by ERα. Even though we do not yet have direct evidence that the observed E2 effects on cell structure are mediated by activation of the Shc and MAPK pathway, the role of Shc and MAPK on cell adhesion and motility has been demonstrated in several cell lines (51, 52). The interaction of Shc with membrane integrin in MCF-7 cells was reported to regulate the locomotion and cell adhesion of MCF-7 cells (53). We postulate that the E2-induced membrane signaling pathway activation, especially ERα-involved activation of Shc, might be linked to cell morphology changes in our cell models.

The suggestion that ERα can reside in or near the cell membrane has been controversial in the past (54). Recent transfection studies provided additional support that ERα can be present in the cell membrane (55). Razandi *et al.* (55) transfected ERα into Chinese hamster ovary (CHO) cells and demonstrated that 3% of expressed ERα was associated with cell membranes. Immunochemical studies with several anti-ERα antibodies on fixed cells including MCF-7 cells also suggested the presence of membrane ERα (20). Our observation using confocal microscopy showed that E2 increased the amounts of ERα in the region of the cell membrane. Our findings provide the first evidence that E2 can functionally influence the amount of ERα translocated into the perimembranous area. At the present time, we do not have direct morphological evidence that Shc is associated with this membrane-associated ERα, but we plan to examine this issue in the future.

In conclusion, we demonstrated that the signaling pathway mediating the rapid action of E2 involved the interaction of Shc with ERα, Shc activation, and the phosphorylation of MAPK. The PTB and SH2 domains of Shc as well as AF-1 domain of ERα are the sites mediating the interaction. Using selective pathway inhibitors and a dominant negative Shc mutant, we confirmed that ERα-mediated Shc activation resulted in MAPK phosphorylation in MCF-7 cells. As evidence of functionality, E2 rapidly stimulated cell morphological changes and induced activation of the MAPK substrate, Elk-1. Additional studies are now required to provide further proof of the role of each of the signaling molecules in the biological events observed.

MATERIALS AND METHODS

Reagents and Plasmids

Tissue culture supplies were obtained from Fisher Scientific (Pittsburgh, PA). Improved MEM (IMEM) with or without phenol red (zinc option, Richter's modification) and FBS were products of Life Technologies, Inc. (Gaithersburg, MD). E2 was obtained from Steraloids (Wilton, NH). ICI was from AstraZeneca, Inc. (Wilmington, DE). E2 and ICI were dissolved in ethanol with a final concentration of ethanol in medium of less than 0.01%. PP2 and PD98059 were from Calbiochem (La Jolla, CA) and dissolved in dimethylsulfoxide. Full-length ERα recombinant protein was purchased from Affinity BioReagents, Inc. (Golden, CO). Protease inhibitors, leupeptin, pepstatin, and aprotinin, were purchased from Sigma (St. Louis, MO).

Mammalian expression vectors encoding human 52-kDa forms or partial regions of Shc with GST fusion proteins were gifts from Dr. Ravichandran Kodi (University of Virginia, Charlottesville, VA) and have been described previously (46). The

amino acid sequences encoded by the different Shc constructs are: full-length (1–471), PTB (17–203), CH (233–377), and SH2 (377–471). The full-length human ERα expression vector (HEGO) was a gift from Dr. Rosalie Uht (University of Virginia) and has been previously reported (56). The ERα expression vectors of AF-1, AF-2, and the Y537F mutant in which tyrosine 537 was mutated into phenylalanine were constructed in our laboratory by PCR and site-directed mutagenesis (QuickChange, Stratagene, La Jolla, CA) as previously described (57). The fusion gene GAL4-Elk-1 and its reporter gene GAL4-E1B-Luc were generously provided by Dr. Michael J. Weber (University of Virginia) and have been described previously (40).

Cell Culture

The MCF-7 cells and COS-1 cells were maintained in 5% FBS-IMEM and 10% FBS-DMEM, respectively. The LTED cells were developed from MCF-7 cells by growing long term (*i.e.* 6 months to 2 yr) in phenol red-free IMEM supplemented with 5% Dextran-coated charcoal-stripped FBS and have been extensively characterized with respect to growth characteristics, responsiveness to E2, and ER content (33, 50). As determined by ligand binding assays as well as mRNA and Western blots, these cells contain 5- to 10-fold higher levels of endogenous ERα than the parental MCF-7 cells. The content of ERβ is negligible to absent as demonstrated by Western blot and PCR (data not shown). All cell lines were routinely cultured in a humidified 95% air-5% CO_2 incubator at 37 C.

Cell Stimulation and Lysate Preparation

For rapid E2-induced signaling pathway studies, cells cultured in IMEM containing 1% dextran-coated charcoal-stripped FBS were treated at 37 C with vehicle or E2. Time and doses are indicated in the figure legends. If inhibitors were used, cells were pretreated with the inhibitors for 30 min and then challenged with E2. Cells were washed once with ice-cold PBS containing 1 mM Na_2VO_4 and extracted with binding buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 5% glycerol, 1% Triton X-100, 25 mM NaF, 2 mM Na_2VO_4, and 10 μg/ml of each aprotinin, leupeptin, and pepstatin). Cell lysates were centrifuged at 14,000 × *g* for 10 min at 4 C to pellet insoluble material. The supernatant of cell extract was analyzed for protein concentration by a DC protein assay kit based on the Lowry method (Bio-Rad Laboratories, Inc.).

Immunoprecipitation and Immunoblotting

Immunoprecipitation and immunoblotting were carried out as described previously (58). Briefly, 1 mg of cell lysate from each treatment was immunoprecipitated using one of the following antibodies: 1 μg monoclonal anti-Shc antibody (PG-797; Santa Cruz Biotechnology, Inc.), 1 μg monoclonal anti-ERα antibody (D-12, Santa Cruz Biotechnology, Inc.), 1.2 μg polyclonal anti-Grb2 antibody (C-23, Santa Cruz Biotechnology, Inc.), 1 μg polyclonal anti-GST antibody, and 1 μg monoclonal anti-Sos antibody (Transduction Laboratories, Inc., Lexington, KY). Incubations proceeded for 4 h at room temperature or overnight at 4 C in the presence of 35 μl of 50% slurry protein-G or protein-A Sepharose beads (Life Technologies, Inc.). The beads were washed three times in cold binding buffer. The proteins eluted from the beads were analyzed on 10% SDS-polyacrylamide gels and transferred to PVDF membranes. The PVDF membranes were probed with one of the following primary antibodies: horseradish peroxidase-conjugated monoclonal antiphosphotyrosine antibody (PY20, Transduction Laboratories, Inc.), polyclonal anti-ERα antibody (HC-20, Santa Cruz Biotechnology, Inc.), polyclonal anti-Shc (Transduction Laboratories, Inc.), polyclonal anti-GST antibodies (Z-5, Santa Cruz Biotechnology, Inc.), monoclonal anti-Grb2 (Transduction Laboratories, Inc.), monoclonal anti-GST, monoclonal anti-ERα N-terminal antibody (COIM2133, Fisher Scientific), polyclonal anti-C-terminal ERα antibodies (MC-20, Santa Cruz Biotechnology, Inc.), and polyclonal anti-Sos antibodies (Transduction Laboratories). After washing the PVDF membrane, the immunoblots were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h [donkey against rabbit IgG from Pierce Chemical Co. (Rockford, IL) or sheep against mouse IgG from Amersham Pharmacia Biotech (Piscataway, NJ)] and further developed using the chemiluminescence detection system (Pierce Chemical Co.). The reciprocal studies for all protein-protein interactions were conducted and the same results were confirmed. All experiments were done at least three times.

GST Pull-Down Assay

Prokaryotic vectors expressing both PTB+CH and SH2 domains of Shc were gifts from Dr. Ravichandran Kodi (University of Virginia), expressed in *Escherichia coli* BL21 and purified with a GST-glutathione affinity system (Amersham Pharmacia Biotech). GST, GST-ShcPTB+CH, and GST-ShcSH2 proteins were induced, solubilized, and bound to Glutathione beads. To test the protein interaction, 15 μl of the suspension were incubated in binding buffer with 5 ng of human full-length ERα expressed and purified from Baculovirus. After incubation, the beads were washed three times with the buffer. Bound proteins were eluted with 40 μl of 2× SDS-PAGE buffer, electrophoretically separated in 10% SDS gel, and transferred on the PVDF membrane. The membrane was probed with monoclonal anti-ERα antibody, and the immunoprotein bands were visualized as described above.

Electroporation and Transfection

COS-1 cells were electroporated exactly as described by Roy *et al.* (59) with 5 μg each of Shc constructs and 5 μg of each ERα-expressing vectors. Cells were then incubated in DMEM supplemented with 10% FBS for 48 h. The medium was changed again with serum-free and phenol-free DMEM for 1 d. Four hours before E2 treatment, the medium was changed again with fresh serum-free and phenol-free medium. Cells were then treated with E2 for 5 min and extracted with binding buffer. At this step, the samples were either stored at −80 C or prepared for protein-protein association assay. To study MAPK-mediated Elk-1 activation, MCF-7 cells were transiently transfected with both 2 ng of GAL4-Elk-1 and 0.1 μg of GAL4-E1B-Luc reporter vectors encoding GAL-Elk fusion protein and GAL-luciferase, respectively. Transfection was performed with Effectene transfection reagent (QIAGEN, Valencia, CA) according to company specifications; 24 h after transfection, cells were treated with different doses of E2 for 6 h. Luciferase activity was measured for each lysates, and samples were normalized by β-galactosidase cotransfected with above vectors. All transfections were conducted at least three times and the data are presented as mean ± SD.

Confocal Analysis of E2-Induced Cell Morphology Changes

Cells were fixed in paraformaldehyde followed by chilled acetone and blocked in normal goat serum followed by incubation with monoclonal antibody against vinculin (Sigma) and with polyclonal antibodies against ERα (Santa Cruz Biotechnology, Inc.). For triple costaining of filamentous actin, ERα and vinculin and 0.1 μM Alexa 546-conjugated phalloidin were included during incubation with the Alexa 633-goat antimouse and Alexa 488 goat antirabbit secondary antibodies. Slides were mounted, and analyzed by a LSM inverted microscope (Carl Zeiss, Thornwood, NY) (60). Each image

represents Z sections at the same cellular level and magnification. Confocal analysis was performed using a Carl Zeiss laser scanning confocal microscope and established methods, involving processing of the same section for each detector and comparing pixel by pixel. Localization of ERα with ruffles is demonstrated by the development of *yellow color* due to *red* and *green* overlapped pixels. The effect of E2 on the changes of filamentous actin in terms of dynamic membrane was measured based on the formation of ruffles and pseudopodia from 12 cells in each treatment. Extensive application of identical methods for analysis of effects of heregulin has been recently published by Adam *et al.* (41).

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Membrane association of estrogen receptor α mediates estrogen effect on MAPK activation☆

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Abstract

Estrogen rapidly activates MAPK in many cell types but the mechanisms have not been fully understood. We previously demonstrated that 17-β-estradiol (estradiol) rapidly induced membrane translocation of estrogen receptor α (ERα) and activated MAPK in MCF-7 breast cancer cells. This study further determines the cause and effect relationship between the presence of membrane ERα and MAPK activation. ERα with a membrane localization signal (HE241G-mem) was expressed and compared with the ones in nucleus (HEGO) or cytosol (HE241G) localization. Confocal microscopy showed that HE241G-mem was expressed in the cell membrane as well as in the cytosol in COS-1 cells. HE241G localized in the cytosol and HEGO in the nucleus. Functional studies showed that only membrane ERα, not cytosol and nuclear ones, responded to estradiol by inducing MAPK phosphorylation. HE241G-mem neither increased basal nor estradiol-induced ERE promoter activation, indicating no transcriptional action involved. Our data support the view that membrane-associated ERα is critical in estrogen-initiated MAPK activation.

Keywords: Estrogen; Estrogen receptor; Membrane; MAPK and signal transduction

Estrogen has an important role in the genesis of human breast cancer and in the growth of established tumors [1]. Classical theory holds that estrogen elicits genomic effects on transcription via nuclear α and β estrogen receptors (ERα and ERβ). As ligand-dependent transcriptional factors, the hormone-bound ERs interact with estrogen response elements (ERE) to activate cell cycle-related immediate early genes in estrogen-responsive tissues and stimulate cancer cell proliferation [2]. This genomic action of estrogen is characterized by delayed onset of action and sensitivity to inhibitors of transcription and translation. However, estrogen, like many cytokines, also appears capable of bypassing genomic signaling pathways and directly regulating many biological actions [3,4]. Such effects have been demonstrated in a variety of cell types with rapid responses to estrogen, within seconds to minutes. Examples of the rapid non-genomic actions of estrogen include activation of Ras [5], Raf-1 [6], protein kinase C [7], protein kinase A [8], Maxi-K channels [9], increments in intracellular calcium levels [10], and an increase of nitric oxide [10]. Such actions may be mediated by a plasma membrane-associated estrogen receptor. However, current evidence that membrane receptors act independently of transcriptional events is inferential and based upon the rapidity of effects observed. No direct experiments have yet demonstrated a requirement for membrane as opposed to cytosolic or nuclear receptors.

A comprehensive understanding of non-genomic receptor functions of 17β-estradiol (estradiol) is currently

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Abbreviations: AF-1, activation function-1; AF-2, activation function-2; DMEM, Dulbecco's modified Eagle's media; DCC medium, dextran coated charcoal stripped medium; DBD, DNA binding domain; ERE, estrogen receptor response element; ERα, estrogen receptor α; FBS, fetal bovine serum; HBD, hormone binding domain; MLS, membrane localization signal; MAPK, mitogen-activated protein kinase; NLS, nuclear localization signal.

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lacking but several biological estrogen actions mediated by non-nuclear ERα have been described. Using anti-ERα antibody, a membrane ERα was demonstrated to mediate the rapid secretion of prolactin in pituitary tumor cells [11]. Expression of the E-domain of ERα targeted to the HeLa cell membrane modulates the anti-apoptotic actions of estradiol [12]. NIH3T3 cells transfected with transcriptional activity-dead ERα can respond to estradiol with increased DNA synthesis [3]. We previously demonstrated that estradiol rapidly activated mitogen-activated protein kinase (MAPK) and induced the association of ERα with plasma membrane in MCF-7 human breast cancer cells [13].

MAPK is a common downstream pathway of many growth factor signal cascades and is essential for the triggering of cell proliferation and differentiation [14]. ERα associated with the cell membrane might be involved in mediating estrogen action on MAPK activation, leading to cell proliferation [15–17]. MAPK belongs to the serine–threonine kinase family and is activated by multiple mitogens. It is important in the regulation of cell differentiation, proliferation, and anti-apoptosis in many cell and tissue types [18,19]. Direct phosphorylation of ERα by MAPK has been reported to potentiate estrogen-mediated gene transactivation [20]. MAPK activities are also up-regulated in many estrogen receptor positive breast cancer cell lines and tissues [21], indicating that MAPK is closely regulated by the action of estrogen.

Prior studies of the possible role of membrane ERα species inferred that the rapid action of estradiol must emanate from membrane-mediated events. We believe that a more direct approach using ERα specifically targeted to the cell membrane is necessary to establish causality. The effects of such a construct can then be compared with wild type ERα and with an ERα residing exclusively in the cytosol. In the present study, we expressed ERα in the nucleus, cytosol, and membrane of COS-1 cells, and then determined which constructs could mediate estradiol rapid action. We directly demonstrated for the first time that ERα in the cell membrane could mediate estradiol-induced MAPK phosphorylation.

Materials and methods

Plasmid constructs. The wild type human ERα expression vector (HEGO) consists of the full-length ERα cDNA fused with the SV40 early promoter and expressed in the pSG5 vector. HE241G is an ERα mutant that lacks a nuclear translocation signal (NLS). The three nuclear localization signals contained within amino acids 256–303 have been deleted, but otherwise the ERα-expressing vector (HE241G/pSG5) is identical to HEGO/pSG5. Both were gifts from Dr. Pierre Chambon (Institute de Chimie Biologique, France) and were described previously [22]. To generate HE241G-mem vector, the fragments of HE241G and a membrane localization signal (MLS) sequence of GAP-43 protein were cloned into the pSG5 vector. The MLS fragment of GAP-43 protein was generated by PCR with the following primers: forward primer with an *Age*I linker: 5′-GCCACCGGTCGCCAC CATGCTGTGCTGT-3′, reverse primer with a *Bgl*II linker and the stop codon: 5′-CAGATCTTCAGATCTTTTGGTCCTCATCATT-3′, using pECFP-mem vector as a template (CLONTECH, Palo Alto, CA). The HE241G fragment was generated by PCR with the following primers: forward primer with an *Eco*R1 linker: 5′-CGGAATTCACCA TGACCATGACCCTCCACA-3′, reverse primer with an *Age*I linker and a mutated stop codon: 5′-GCCACCGGTCTG ACTGTGGCAG GGAAACCCTC-3′, using HE241G/pSG5 as a template. The vector was prepared by cutting HE241G/pSG5 with *Eco*RI/*Bgl*II. The PCR products, HE241G and MLS sequence, were digested with *Eco*R1/*Age*I and *Age*I/*Bgl*II separately, and then all three fragments were ligated into pSG5 plasmid. The sequence of the construct was verified by DNA sequencing.

Cell culture and transfection. COS-1 cells obtained from ATCC (Manassas, VA) were routinely maintained in DMEM media supplemented with 5% fetal bovine serum (FBS) at 37 °C under 5% CO_2. For all experiments unless specified, cells were seeded in regular DMEM supplemented with 5% FBS (Gibco Life Technologies, Rockville, MD) in 6-well plates and allowed to grow to 40% confluence. The medium was changed into phenol red free DMEM containing 5% dextran-coated charcoal-stripped (DCC) FBS. DCC-FBS was prepared as described previously [23]. Cells were then transiently transfected with 5 μg plasmid DNA of either empty vector (pSG5), HEGO, HE241G, or HE241G-mem using the Effectene reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Cells were treated with vehicle or 17β-estradiol (Steraloids, Wilton, NH) for the times indicated in the figures. Transfection efficiency was monitored by staining cells with β-galactosidase and counting stained cells.

Confocal microscopy. The confocal microscopic method of studying ERα localization has been reported before [13]. Briefly, cells on glass coverslips were fixed in 4% paraformaldehyde in PBS and then permeabilized with 0.15 M sucrose in the presence of 0.2% Triton X-100 for 5 min. The fixed cells were first incubated in blocking buffer [PBS containing 3% normal goat serum (G-9023; Sigma)] for 2 h and then stained for 60 min with a monoclonal anti-ERα antibody (D-12, Santa Cruz, CA) diluted to a final concentration of 3 μg/ml in PBS containing 0.5% BSA, 0.1% goat serum, and 0.3% Tween 20. Normal rabbit IgG at the same concentration was used as a control. For double co-staining of filamentous actin, 0.1 μM Alexa 546-conjugated phalloidin was included during the incubation with the Alexa 633-goat anti-mouse secondary antibodies in a 1:200 dilution. For each treatment, representative fluorescence images were recorded using a Zeiss-LSM inverted microscope equipped with a confocal function. Each image represents Z sections at the same cellular level and magnification. Confocal analysis was performed using a Zeiss laser scanning confocal microscope, involving processing of the same section for each detector and comparing pixel by pixel. Localization of ERα with ruffles is demonstrated by the development of yellow color due to red and green overlapped pixels. Images from all treatment groups were captured at the same time using identical image capture parameters.

Western blot for MAPK phosphorylation study. The method for detection of phosphorylated MAPK has been reported before [13]. Briefly, cells cultured in DMEM containing 1% DCC-FBS were treated at 37 °C with vehicle or estradiol. Times and doses are indicated in the figure legends. At the end of each experiment, cells were washed once with ice-cold phosphate-buffered saline containing 1 mM Na_2VO_4 and extracted with binding buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 5% glycerol, 1% Triton X-100, 25 mM NaF, 2 mM Na_2VO_4, 10 μg/ml of each aprotinin, leupeptin, and pepstatin). Cell lysates were centrifuged at 14,000*g* for 10 min at 4 °C to pellet insoluble material. The supernatant of the cell extract was analyzed for protein concentration by a DC protein assay kit based on the Lowry method (Bio-Rad Laboratories, Hercules, CA). Activated MAPK was detected using anti-dual phosphotyrosine MAPK antibody (9101S, Cell Signaling Tech, Beverly, MA) and the MAPK protein loading was

detected using a polyclonal anti-MAPK antibody (K-23, Santa Cruz Biotech., CA). The immunoblot was further developed using a chemiluminescence detection system (PIERCE, Rockford, IL).

Assay of ERE promoter activity. To study ERE promoter activation, 0.5 μg of β-galactosidase and ERE-tk-luciferase reporter plasmids were co-transfected into the cells with different ERα expressing vectors. Cells were than treated with vehicle or estradiol for 6 h and extracted with luciferase assay buffer provided with the kit (E4030, Promega, Madison, WI). The activities for both luciferase and β-galactosidase were assayed using a kit according to the manufacturer's instructions. Results for ERE promoter assay are expressed as means ± SEM. A two-tailed unpaired Student's t test was used for statistical analysis. The difference between experimental groups was considered significant when the p value was < 0.05 (*).

Results

Intracellular distribution of ERα in COS-1 cells

Our initial goal was to create "designer" cells in which the ERα would localize specifically to the cell membrane, cytoplasm, and nucleus (Fig. 1). To accomplish this, we transiently transfected COS-1 cells with three different ERα constructs: HEGO (wild type), HE241G (NLS deleted), and HE241G-mem (MLS ligated onto the C-terminal of HE241G). We then utilized confocal fluorescence microscopy to observe precisely where the various receptor subtypes localized. As shown in Fig. 2, the cell membrane is stained red by interaction with phalloidin which binds to membrane actin (Figs. 2A, D, and G). Green stains show the localization of the ERα proteins (Figs. 2B, E, and H). Overlaying both green and red images forms a yellow color and allows demonstration of co-localization (Figs. 2C, F, and I). Using this methodology, we demonstrated that wild type ERα (HEGO) localized nearly exclusively in the nucleus of COS-1 cells (Fig. 2C). HE241G-mem ERα was distributed in both membrane (arrow) and cytosol (Fig. 2I) and HE241G mainly in cytosol (Fig. 2F). Quantitative morphometric analysis of different ERα distributions is shown in Table 1. 96% of HEGO localized to the nucleus, but only 3% to the cytosol and less than 0.5% to the plasma membrane. 94% of HE241G ERα, which has the NLS deleted, was found in the cytosol as expected [22] versus 3.9% in the nucleus and 2.3% in the plasma membrane. 36% of HE241G-mem ERα, which contains MLS but not NLS, localizes along the plasma membrane as well as in the cytosol (64%). Taken together, our findings demonstrate the ability to direct ERα away from the nucleus and to the cytosol and cell membrane. We did not achieve exclusive membrane localization with HE241G-mem, which is in agreement with the previous report [24]. Nonetheless, this model system allowed us to demonstrate the separate effects of the nuclear, cytosolic, and membrane ERα by subtracting the effects induced by receptor localizing exclusively to the cytosol from those caused by the combination of plasma membrane and cytosol receptor.

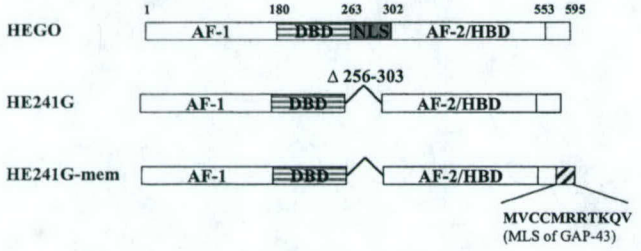

Fig. 1. Schematic illustration of ERα constructs. HEGO is a wild type ERα-expressing vector that encodes a 595 amino acid protein. The activation function 1 (AF-1), DNA binding domain (DBD), nuclear localization sequence (NLS), and activation function 2/hormone binding domains (AF-2/HBD) are shown. HE241G encodes a mutated ERα which has the NLS deleted (Δ256–303). HE241G-mem (membrane) was constructed by ligation of a membrane localization sequence (MLS) of GAP-43 on the C-termini of HE241G. The MLS is illustrated by a dashed box and the eleven amino acid sequence of the MLS is shown under the box.

Membrane-associated ERα mediating estradiol effect on MAPK phosphorylation

Our previous studies reported that estradiol rapidly induced MAPK phosphorylation and provided indirect evidence that this process may involve a subset of ERα molecules present on the cell membrane [13]. The present study sought to directly demonstrate the requirement for membrane ERα on MAPK activation. In this experiment, COS-1 cells, which do not express endogenous ERα, were transiently transfected with pSG5 empty vector or the different ERα expressing vectors, HEGO, HE241G, and HE241G-mem. MAPK phosphorylation was assayed after estradiol treatment. As shown in Fig. 3A (top panel), 15 min treatment with 10 nM estradiol greatly stimulated MAPK phosphorylation in cells transfected with HE241G-mem. In marked contrast, cells transfected with empty vector pSG5, the wild type estrogen receptor (HEGO), and HE241G ERα did not show any estradiol-induced increase in the phosphorylation of MAPK. Equal amounts of MAPK protein loading were confirmed (Fig. 3A, middle panels). Equivalent expression of wild type and mutant ERα proteins in COS-1 cells was shown (lower panel, Fig. 3A). Both HE241G and HE241G-mem have NLS deleted and are smaller molecular weight proteins, showing faster migration than HEGO ERα. Furthermore, the estradiol-induced MAPK phosphorylation mediated by HE241G-mem occurred in a time- and dose-dependent manner, with the time to respond to estradiol as early as 3 min (top panel, Fig. 3B) and optimal dose at 10 nM (top panel, Fig. 3C). Equal amounts of MAPK protein loading were shown (bottom panels, Figs. 3B and C). Our results indicate that ERα membrane association was necessary for estradiol to activate MAPK in this model system.

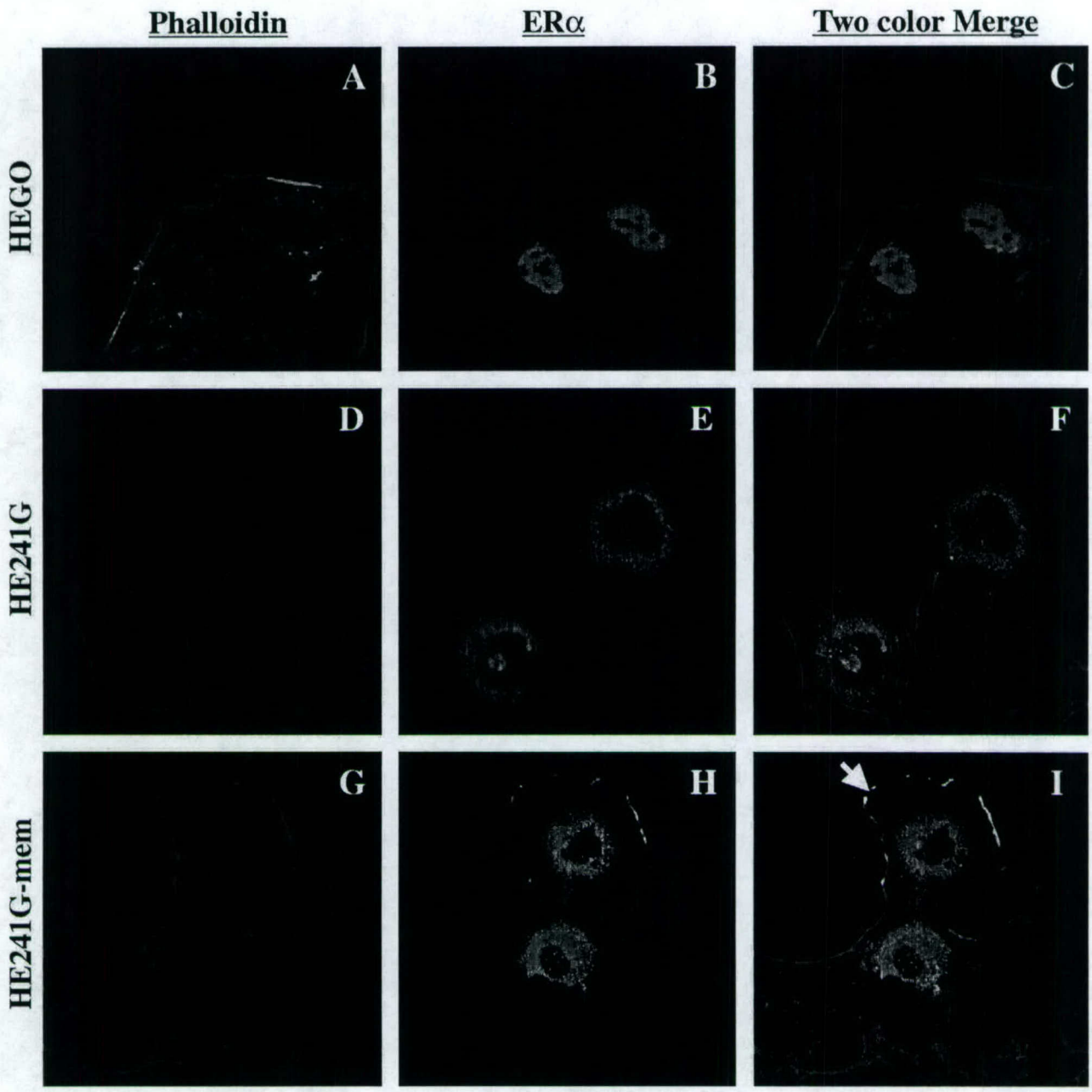

Fig. 2. Confocal microscopy analysis of ERα subcellular localization. COS-1 cells were transfected with HEGO, HE241G, and HE241G-mem vectors. ERα and cell membrane were identified by immunofluorescence staining of ERα with monoclonal anti-ERα antibody and fluorescence staining of actin with phalloidin. The confocal microscope was used to observe ER subcellular distribution. Red fluorescent color in the left column indicates filamentous actin stained by phalloidin and the middle column shows the ERα stain (green color). The two color-merged (ERα/filamentous actin) view is shown on the right column. The top panels illustrate ERα expression in COS-1 cells transfected with HEGO. The middle panels are cells transfected with HE241G and lower panels HE241G-mem.

Effect of estradiol on ERE promoter activation

We reasoned that small amounts of ERα could potentially enter the nucleus even without a localization signal and yet escape detection by immunofluorescence. To rule out this confounding problem, we assessed ERα-mediated transcriptional activation by measuring ERE promoter activation as a highly sensitive indicator of the presence of nuclear ER. In essence, this reporter assay provided a very sensitive means of determining whether any 241-G and 241G-mem entered the nucleus even though this was not detected by confocal microscopy. Accordingly, we co-transfected COS-1 cells with an ERE-tk-luciferase reporter gene and either pSG5, HEGO, HE241G, or HE241G-mem ERα expressing vectors. In our experiments, β-galactosidase activities

Table 1
Quantitation of ERα cellular localization in COS-1 cells

	HEGO		HE241G		HE241G-mem	
	% of cells[a]	Index[b]	% of cells	Index	% of cells	Index
Membrane	0.775	0.42	4.3	2.3	35	35.5
Cytosol	4.7	3.1	90.8	93.8	65	64.1
Nucleus	94	96	4.9	3.9	0	0

The subcellular location was counted regardless of other location expression.
[a] Percentage of cells calculated from at least 100 ERα positive stained cells transfected with indicated vector.
[b] Calculated by semi-quantitation of ERα expression in a four level scale (+ to ++++). The index was calculated by the formula: the score of semi-quantitation × no. of transfected cells of the same scale × 100.

were used as internal controls for transfection efficiency. As shown in Fig. 4, a very low basal level of luciferase activity was observed in HE241G-mem transfected COS-1 cells, indicating that minimal or no ERα mutant receptor was localized in the nucleus. A 2-fold elevated basal level of luciferase activities was shown in HE241G-transfected cells, closely correlated with a small portion of HE241G in the nucleus. Neither HE241G-mem nor HE241G responded to estradiol with activation of luciferase activities (Fig. 4). As a positive control, estradiol induced a 8-fold increase of luciferase activity in the cells transfected with HEGO ($P < .001$), suggesting that nuclear ERα is required for ERE promoter activation. Dose response experiments with estradiol showed maximum effects at 10 nM (data not shown). These studies provided further proof that HE241G-mem acts via cell membrane and not nuclear effects.

Discussion

The present study investigated whether cell membrane-associated ERα might be capable of mediating estradiol stimulation of MAPK activation. By using estrogen receptors specifically targeted to nucleus, cytosol, and plasma membrane, we directly demonstrated the functionality of plasma membrane ERα with respect to MAPK activation. As tools, we utilized two ERα constructs lacking nuclear localization signals. Key to the critical interpretation of these data was the demonstration that these constructs did not enter the nucleus in functionally significant amounts. Two methods, confocal microscopic visualization and assessment of function with a reporter of nuclear transcriptional activity, confirmed the lack of nuclear functionality of the ERα constructs without nuclear localization signals.

A second key component of our study is the ability to distinguish plasma membrane effects from cytosolic effects of ERα. This was accomplished by subtracting the effects exerted by the cytosol ERα from those induced by an ERα localizing to both cytosol and plasma membrane. One of these constructs (HE241G) mainly localized in the cytosol and the other (HE242G-mem) distributed to both cytosol and membrane. The receptor located exclusively in the cytosol exerted no estradiol-stimulated effects on MAPK. In contrast, the receptor localized in both cell membrane and cytosol mediated the effect of estradiol to induce to MAPK phosphorylation. By comparing differential effects, we could infer that it was the membrane-targeted receptor that induced the MAPK activation. This methodology allowed us to show that ERα membrane association is required for estradiol to activate MAPK in this system. Notably these observations provide robust proof that estradiol can initiate a cell membrane-mediated event through interaction with a membrane-localized ERα. The validity of our conclusion is strengthened by the use of an important control; the wild type ERα that localizes almost exclusively to the nucleus of the COS-1 cells has no effect on MAPK activation. Taken together, these results can be interpreted as proof of the principle that membrane-associated ERα can mediate the action of estradiol, since neither nuclear nor cytoplasmic ERα allowed estradiol-induced MAPK phosphorylation.

Increasing evidence has shown that estradiol can induce MAPK phosphorylation in MCF-7 cells [5,13,25]. Recently we demonstrated for the first time that translocation of ERα to the membrane occurred following estradiol treatment in the same time frame as MAPK activation [13]. Interestingly the ERα in MCF-7 cells is wild type and found in both the membrane and the nucleus [11,13]. It is presumed, but not yet proven, that wild type cells have some means of translocating the ERα into the cell membrane. We postulate that some membrane proteins that physically associate with ERα might be required for ER membrane association. Candidates for this function include Shc, Src, IGF-1 receptor, caveolin, and PI3 kinase [13,26–28]. Clearly, such mechanisms are not present in COS-1 cells since neither HEGO nor HE241G ERα translocate to the membrane before or upon estradiol stimulation (data not shown). Consequently, the model described in this manuscript provides a more direct means of testing effects of membrane ERα because no dynamic processes are necessary to bring it into the membrane. Such inducible mechanisms would be expected to confound interpretation of the results.

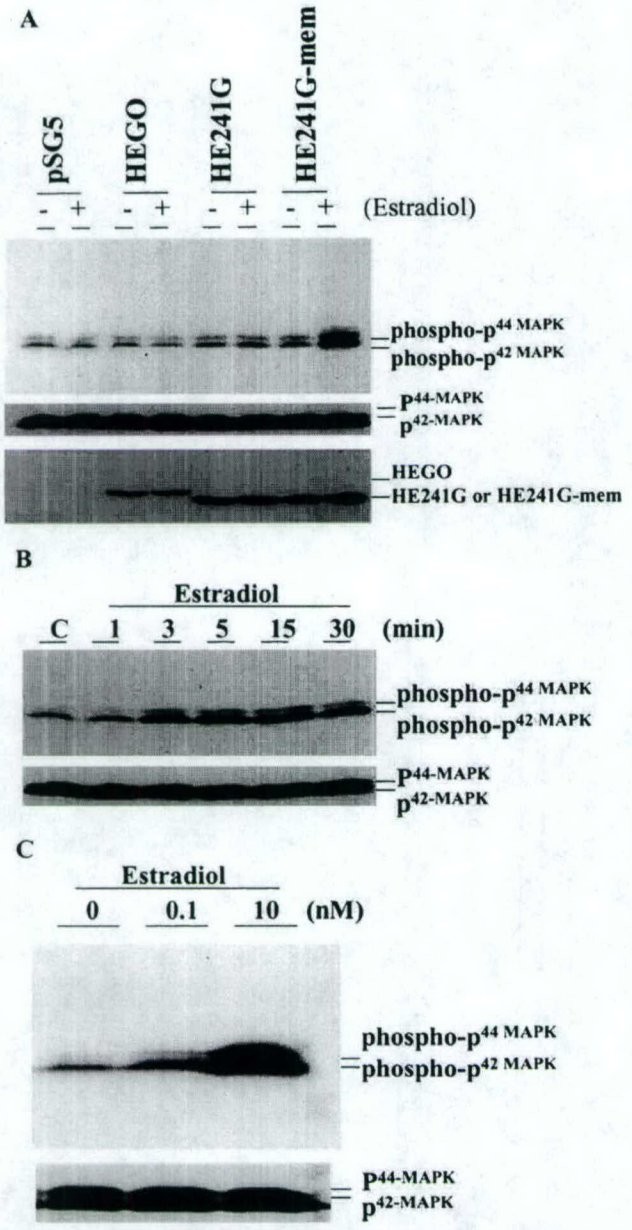

Fig. 3. Estradiol effect on MAPK phosphorylation in COS-1 cells transfected with ERα constructs. COS-1 cells were transiently transfected with different ERα-expressing vectors as indicated (A) or only HE241G-mem (B and C) for two days and then treated with 10 nM estradiol for 15 min (A) or for different times and doses as indicated (B and C). The phosphorylation status of MAPK (p42 and p44) from whole cell lysates was assessed using an antibody recognizing dual phosphorylated MAPK (top panels). Total MAPK protein loading (A, B, and C) and ERα expression in COS-1 cells (A) were determined by re-probing the PVDF membrane with anti-total MAPK antibody (bottom panels). The positions of phosphorylated and total MAPK proteins are indicated at the right. The experiment was done four times and a representative result is shown.

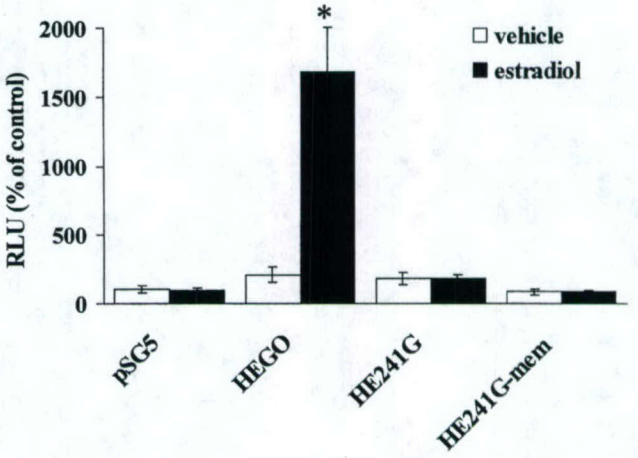

Fig. 4. Effect of estradiol on ERE promoter activation. COS-1 cells were transiently co-transfected with different ERα expressing vectors plus ERE-tk-luciferase and β-galactosidase plasmids. After 24 h, cells were treated with vehicle or 10 nM estradiol for 6 h. Both luciferase and β-gal activities were measured as described in Materials and methods. The luciferase activities were normalized by β-gal activities and expressed as the relative luciferase units (RLU).

Regardless of the considerations discussed above, further work is necessary to determine just how the ERα is localized in the cell membrane under normal conditions and whether membrane-associated ERα can mediate the biological effects of estradiol on cell proliferation and apoptosis. Currently, the mechanism whereby membrane-associated ERα (HE241G-mem) mediates estradiol action on MAPK phosphorylation is not clear and is under active investigation.

Because of the multiple effects of estradiol, standard approaches cannot be used to distinguish the involvement of genomic actions from non-genomic pathways or effects mediated by cross talk between these pathways in ERα-expressing cells. We reasoned that the best approach to distinguish these effects was to utilize ERα mutants with specific localization properties in non-ERα bearing cells.To do this, we constructed an ERα with its NLS-deleted and an eleven amino acid MLS sequence of the GAP-43 protein ligated on its C-termini [29]. GAP-43, also named neuromodulin, is a neurospecific calmodulin binding protein tightly bound to the cell membrane [30]. It has been demonstrated that an eleven amino acid sequence, MLCCMRRTK of neuromodulin, was able to direct the protein membrane association [31]. Experiments conducted in vivo and in vitro demonstrated that a range of 20–60% of GAP-43 is associated with the cell membrane [24,32]. Expressing HE241G-mem, we observed ERα protein distribution in COS-1 cells similar to these reports. Expressing ERα on the cell membrane, we provided proof of the principle that membrane association of ERα can be essential for estradiol to activate MAPK in a model system. We recognize, of course, that our observations do not provide evidence that membrane-mediated effects are essential for MAPK activation under all circumstances.

Our confocal microscopy results show that HE241G-mem ERα is expressed along the cell membrane but the

majority appear to be in the cytosol. Therefore, one might question whether the ERα is functioning in the cytosol to mediate certain effects of estradiol. The HE241G mutant provides important evidence regarding this question. In our system, HE241G ERα is exclusively in the cytosol and does not allow estradiol to increase MAPK. Thus, the membrane portion of HE241G-mem receptor appears to mediate these effects since the cells transfected with cytosolic receptor (HE241G) did not show any response.

Other reported experiments inferred a direct biologic effect of membrane-bound ERα. Estradiol, mediated by a membrane ER, rapidly stimulated prolactin release from GH3/B6 pituitary tumor cells. As evidence of this membrane effect, an antibody against the hinge regions of the ERα (H-151) blocked the stimulation of prolactin [11]. Since the antibody presumably cannot enter the cell, these effects would appear to result from the action of the antibody at the cell surface. However, this experiment is indirect, as it relies on the inference that the antibody or portions do not enter the cytoplasm of the cell. In other experiments, membrane ERα has been reported to mediate estradiol action on anti-apoptotic processes [12,33]. But different mechanisms have also been reported, such as the activation of p38 MAPK and the up-regulation of Bcl-2 gene, an inhibitor of apoptosis [34,35]. The functional role of HE241G-mem on apoptosis was tested in our cell model system using the ELISA method previously employed in our laboratory [36]. No significant anti-apoptotic effect was evidenced in response to estradiol (data not shown). But use of the ELISA method to test the population of transfected cells for apoptosis might not be conclusive and we must examine apoptosis specifically in ERα transfected cells using TUNEL assay.

Regarding the membrane role of ERα, the data of Castoria et al. appear to differ somewhat from ours. Using the selective MAPK pathway inhibitor PD98059, they demonstrated that MAPK activation is involved in estradiol action on NIH 3T3 fibroblastic cells transfected with either HEGO or HE241G ERα [3]. Both NIH 3T3 and HeLa cells were tested in our laboratories but low transfection efficiency precluded interpretation. It is conceivable that NIH 3T3 cells may have a means of transporting both HEGO and HE241G ERα into the cell membrane. Alternatively, this discrepancy could be due to differences in the biologic properties of NIH 3T3 and COS-1 cells. Regardless of this difference, the data from both systems support the conclusion that estradiol may stimulate cell proliferation in the absence of enhanced nuclear transcriptional activity. Taking our data together with theirs, it would appear likely that certain cells have developed mechanisms to direct ERα to the cell membrane.

Membrane ERα, structurally identical to nuclear receptor, has been recently observed in various cells such as human breast cancer MCF-7 cells [37], human monocytes [37], rabbit uterus cells [38], and transfected hamster ovary cells [39]. However, the mechanism for ERα plasma membrane localization is still unknown. Comparing the ERα protein sequence with known membrane localization sequences, such as that in GAP-43 [29] and Ras [40], we did not find consensus sequences within the ERα protein. Further studies beyond the scope of this publication will be required to determine exactly what mechanisms are involved in directing ERα to the cell membrane. It is postulated that either there exist unknown membrane-targeting sequences in the ERα protein or some membrane-targeting chaperone proteins might indirectly mediate this ERα membrane association. At present, no such protein has yet been identified.

In conclusion, we demonstrated that MAPK activation by estradiol requires ERα membrane association in our model system. This membrane-dependent action of estradiol may be crucial to estrogen function in breast cancer cell proliferation and might be one of the key steps in mediating the estrogen-dependent activation of cellular events. The specific mechanisms regulating translocation of ERα from the cytosol to the cell membrane are not currently understood. Use of an ERα-negative cell line, such as MDA-MB-231, will be necessary to further explore the possibility of membrane ERα involvement. Additional studies are now required to provide further proof of the role of membrane ERα in biologic events and mechanisms mediating translocation of native ERα into the membrane.

Acknowledgments

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The role of Shc and insulin-like growth factor 1 receptor in mediating the translocation of estrogen receptor α to the plasma membrane

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Our previous studies demonstrated that 17β-estradiol (E2) rapidly induces the interaction of estrogen receptor α (ERα) with the adapter protein Shc, the translocation of ERα to the cell membrane, and the formation of dynamic membrane structures in MCF-7 breast cancer cells. The present study examined how E2 causes ERα to translocate to the region of the plasma membrane and focused on mechanisms whereby Shc and the insulin-like growth factor-1 receptor (IGF-1R) mediate this process. Shc physically interacts with IGF-1R in the plasma membrane, and E2 activates IGF-1R. We reasoned that ERα, when bound to Shc, would be directed to the region of the plasma membrane by the same processes, causing membrane translocation of Shc. We confirmed that E2 rapidly induced IGF-1R phosphorylation and demonstrated that E2 induced formation of a ternary protein complex among Shc, ERα, and IGF-1R. Knock down of Shc with a specific small inhibitory RNA decreased the association of ERα with IGF-1R by 87%, suggesting that Shc is a crucial molecule in the formation of this ternary complex. Confocal microscopy studies provided further confirmation of the functional roles of Shc and the IGF-1R in the translocation of ERα to the region of the membrane. Down-regulation of Shc, ERα, or IGF-1R with specific small inhibitory RNAs all blocked E2-induced mitogen-activated protein kinase phosphorylation. Together, our results demonstrate that Shc and IGF-1R serve as key elements in the translocation of ERα to the cell membrane and in the facilitation of ERα-mediated rapid E2 action.

Estradiol-17β (E2) plays a prominent role in mediating the maturation, proliferation, and differentiation of the mammary gland and influences the growth and development of breast cancer (1). The biological effects of E2 are mediated through the estrogen receptor (ER) acting via classical genomic events in the nucleus and by rapid nongenomic actions at the plasma membrane. Mounting evidence suggests that the nongenomic actions of E2 involve classical receptors acting at the level of the plasma membrane (2, 3). Although debated previously, current evidence favors the concept that the plasma membrane ER is the same as that locating in the nucleus, but that the membrane pool is present in much lower concentrations (4–6). Using antibodies directed against different epitopes of nuclear ERα, many laboratories, including ours, have demonstrated that the classical ERα can locate in the region of the cell membrane (7–9). However, unlike many growth factor receptors, ERα has no intrinsic transmembrane domain, raising the question how it can translocate to the plasma membrane. As a potential mechanism, we postulated that ERα might bind to Shc, which itself is translocated to the plasma membrane.

Shc binds to docking sites on many growth factor receptors in the plasma membrane, including epidermal growth factor (10), nerve growth factor (11), platelet-derived growth factor (10), and insulin-like growth factor (IGF) receptors (12). The recruitment of Shc is linked to the intracellular domains of these various activated membrane receptors. When these docking sites are activated, they recruit Shc and in turn Grb-2, SOS, RAS, RAF, and mitogen-activated protein kinase (MAPK), leading to MAPK pathway activation. We postulated that the IGF-1 receptor (IGF-1R) might be a major site for the docking of a Shc/ERα complex based on the following evidence: (*i*) E2 stimulates the rapid activation of the IGF-1R through its phosphorylation; (*ii*) ERα and IGF-1R are coexpressed, and the levels of the receptors are positively correlated in many breast cancer cells (13); (*iii*) crosstalk between the two receptor-mediated signaling pathways occurs and is strongly linked to the synergistic induction of cell proliferation and antiapoptosis of breast cancer cells (14); and (*iv*) E2 induces binding of ERα to Shc (9). Taking these data into consideration, we investigated whether Shc acts as a translocator carrying ERα to the region of the plasma membrane and binding to the transmembrane receptor IGF-1R.

Materials and Methods

Cell Culture, Small Inhibitory RNA (siRNA) Transfection, and Immunoblotting. Detailed description of the reagents, tissue culture methods, and immunoblotting techniques has been described in detail (9) and is provided in detail in *Supporting Text*, which is published as supporting information on the PNAS web site. For the siRNA studies, a smart pool of double-stranded siRNA against Shc (P-002031-01-20), ERα (ESR1-NM-00-05), or IGF-1R (IGF1R-NM-000875) as well as nonspecific siRNA (D-001206-01-05) were obtained from Dharmacon Tech (Lafayette, CO) and used according to the manufacturer's instructions (see *Supporting Materials and Methods*). For the study of protein–protein interactions, the reagents and methods to detect Shc and ERα were identical to those previously described (9). IGF-1R phosphorylation and detection were carried out by immunoprecipitation of IGF-1R by using anti-IGF-1R monoclonal antibody (3B7, Santa Cruz Biotechnology) and detection of its phosphorylation status and protein expression by using antiphosphotyrosine antibody (4G10, Upstate Biotechnology, Lake Placid, NY) and anti-IGF-1R β domain antibody (C-20, Santa Cruz Biotechnology), respectively.

Confocal Analysis of Colocalization of ERα, Shc, and IGF-1R on Cell Membrane. Detailed methods regarding confocal microscopic studies were previously described in detail and are provided in *Supporting Materials and Methods* in *Supporting Text* (15). Primary antibodies used were: rabbit anti-ERα (H184, Santa Cruz Biotechnology sc-7207 diluted 1:100), mouse anti-Shc (PG-797, Santa Cruz Biotechnology sc-967 diluted 1:200), mouse anti-IGF-1R (3B7, Santa Cruz Biotechnology sc-967 diluted 1:200), and mouse antivinculin (hVIN-1, Sigma V-9131 diluted 1:200). Anti-mouse and anti-rabbit second antibodies conjugated to the fluorescent dyes Alexa 488 (green) or Alexa 633 (far red, colored blue) and phalloidin conjugated to Alexa 546 (red) were purchased from Molecular Probes. Colocalization of ERα, Shc, and

Abbreviations: E2, 17β-estradiol; EGFR, epidermal growth factor receptor; ERα, estrogen receptor α; IGF-1R, insulin-like growth factor 1 receptor; MAPK, mitogen-activated protein kinase; siRNA, small inhibitory RNA; ICI, ICI 182,780.

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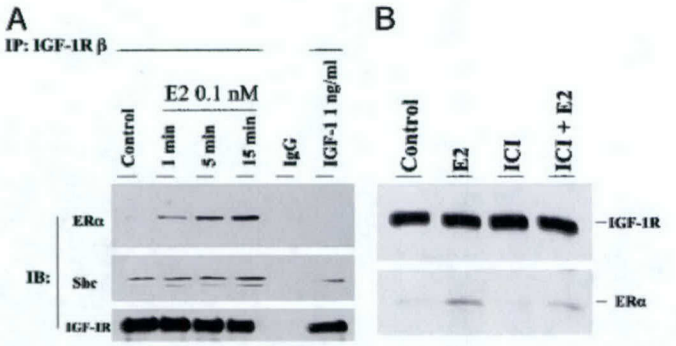

Fig. 1. (*A*) E2-induced protein complex formation among ERα, Shc, and IGF-1R. MCF-7 cells were treated with vehicle, 1 ng/ml IGF-1, or E2 at 0.1 nM for the times indicated. Lysates were immunoprecipitated with IGF-1R antibody. The nonspecific monoclonal antibody (IgG) served as a negative control. (*B*) The effect of ICI on E2-induced protein–protein interaction was assayed by immunoprecipitation of IGF-1R and detection of ERα on Western blot.

IGF-1R on the cell membrane was demonstrated by the development of white color due to the overlapping of green, blue, and red pixels.

Statistical Analysis. All reported values are the means ± SEM. Statistical comparisons were determined with two-tailed Student's *t* tests. Results were considered statistically significant if the *P* value was <0.05.

Results

E2 Stimulates Formation of a Ternary Complex Involving Shc, ERα, and IGF-1R. We previously demonstrated that E2 induces the physical association of Shc with ERα in MCF-7 cells (9). Shc, in turn, binds to the phosphorylated tyrosin residues of IGF-1R (16). From these observations, we postulated that E2-induced ERα membrane translocation might be due to an increased Shc association with membrane receptor IGF-1R. We sought to identify this ternary protein complex by immunoprecipitation of IGF-1R and detection of coimmunoprecipitated Shc and ERα in MCF-7 cells treated with or without E2. Under basal conditions, Shc, but not ERα, associated with IGF-1R (Fig. 1*A*). E2 increased both ERα and Shc association with IGF-1R in a time-dependent fashion at the physiologic concentration of 0.1 nM. In contrast, IGF-1 at the dose used (1 ng/ml) had little effect on the association of ERα with IGF-1R. We further confirmed the formation of the ternary protein complex by using a reciprocal order of immunoprecipitation of Shc and then detection of both IGF-1R and ERα on Western blot (Fig. 6, which is published as supporting information on the PNAS web site). This ERα/Shc/IGF-1R complex is sensitive to disruption by the antiestrogen, ICI 182,780 (ICI), providing additional evidence that activated ERα plays an initiating role in the complex formation (Fig. 1*B*).

Activation of IGF-1R Is Involved in E2 Rapid Action in MCF-7 Cells. The time-dependent effects of E2 on IGF-1R phosphorylation were tested by using cell extracts prepared at various times after treatment. E2 stimulated IGF-1R phosphorylation as early as 3 min with the maximum effect at 15 min (Fig. 7*A*, which is published as supporting information on the PNAS web site). As a positive control, IGF-1 at 1 ng/ml also strongly stimulated IGF-1R phosphorylation in MCF-7 cells. To further study the effects of other signaling molecules on E2-induced IGF-1R activation, we used a series of selective inhibitors, AG1024 for IGF-1R phosphorylation (17), ICI for ERα, and PP2 for Src, respectively. As shown in Fig. 7*B*, which is published as supporting information on the PNAS web site, E2 greatly increased IGF-1R phosphorylation, which was blocked by all three inhibitors, indicating that ERα and c-Src are

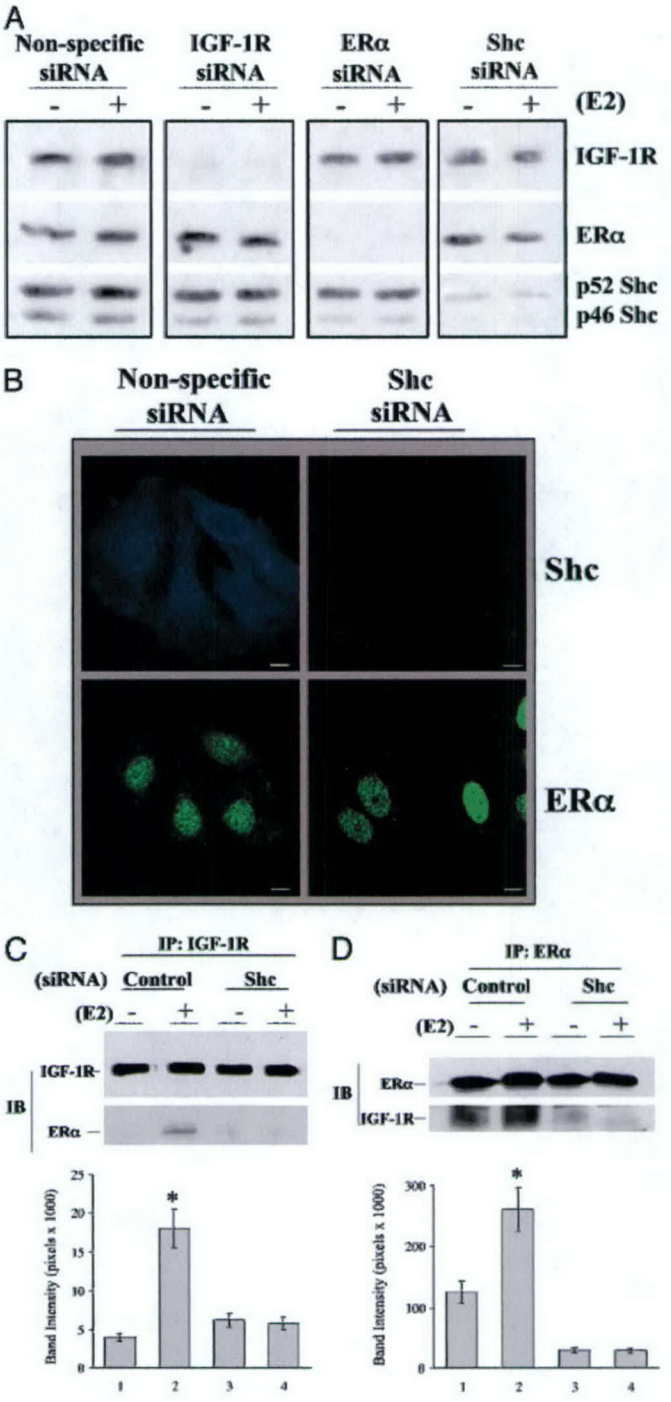

Fig. 2. Protein silencing and the role of Shc in protein complex formation in MCF-7 cells. (*A*) Cells were transfected with nonspecific or specific siRNA for 2 days and then treated with vehicle or 0.1 nM E2 for 15 min for additional MAPK phosphorylation assay. Polyvinylidene difluoride membranes were probed with the specific antibodies to detect the protein expression of IGF-1R, ERα, and Shc. (*B*) Confocal microscopy study of siRNA expression in MCF-7 cells. Cells, transfected with or without siRNA against Shc, were grown on cover slips and then subjected to immunofluorescence staining for Shc (blue) and ERα (green), as described in *Materials and Methods*. (*C* and *D*) The role of Shc in mediating the interaction of ERα and IGF-1R. Cells transfected with siRNA as indicated were treated with vehicle or E2 at 0.1 nM. The IGF-1R and ERα interaction was assayed by immunoprecipitation of IGF-1R and detection of ERα on Western blot (*C*) or by immunoprecipitation of ERα and detection of IGF-1R on Western blot (*D*). The data are from three experiments combined, and only one experiment is shown. *, $P < 0.05$ comparison of E2-treated cells with vehicle control.

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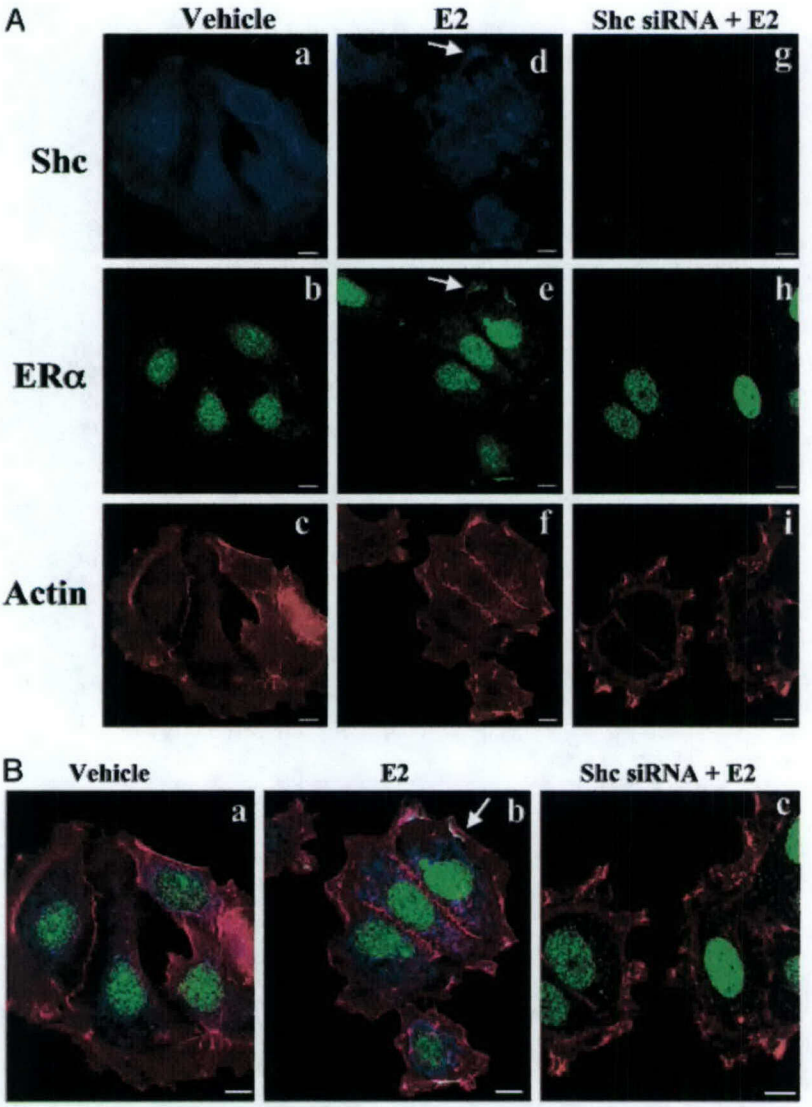

Fig. 3. Shc involvement in E2-induced ERα membrane association in MCF-7 cells. Cells were transfected with or without siRNA directed against Shc. Two days later, cells were treated with vehicle or E2 (0.1 nM) for 15 min and then subjected to immunofluorescence staining with anti-Shc anti-ERα antibodies. Actin was stained with phalloidin, indicating filamentous cell membrane. (*A*) The nonmerged images show Shc in blue, ERα in green, and cell membrane in red. (*Left*) The vehicle-treated cells stained with Shc, ERα, and actin. (*Center* and *Right*) Estrogen-treated cells with or without siRNA of Shc expression. (*B*) Colocalization of ERα, Shc, and actin on cell membrane is shown in three color-merged images. To highlight the specific changes, the arrows illustrate one example of the effects in single cells. Close inspection reveals multiple changes not marked by arrows.

upstream components of IGF-1R activation in E2 rapid action. Taken together, our results demonstrated that IGF-1R is rapidly activated by E2 treatment in MCF-7 cells, a process regulated by ERα and c-Src.

Protein Silencing and the Role of Shc in the Ternary Complex Formation. We determined whether the physical association among Shc, ERα, and IGF-1R was necessary for E2 to exert functional effects and specifically knocked down each component with siRNA technology. When cells were transfected with the siRNA directed against IGF-1R, the levels of this protein were decreased 24–48 h after transfection and reached <5–10% when compared with levels of control proteins (Fig. 2*A*). The siRNA against ERα exerted similar specific effects. When MCF-7 cells were transfected with the siRNA against Shc, the levels of both p52 and p46 isoforms were equally decreased, suggesting that both p52 and p46 mRNA of Shc were equally targeted by the siRNA. Decreased Shc protein expression was documented only in the cells expressing siRNA against Shc but not in cells expressing siRNA against either IGF-1R or ERα, demonstrating the specificity of the siRNA. This was also the case for siRNAs against IGF-1R or ERα. We then used confocal microscopy techniques to demonstrate specificity of knock down of Shc on ERα expression in two-color immunofluorescence staining image (Fig. 2*B*). The result clearly shows that siRNA against Shc only decreased Shc protein level without altering the level of ERα expression, as indicated by nuclear ERα staining. To further confirm the specificity of the siRNAs, we probed the polyvinylidene difluoride membrane with two unrelated proteins, insulin receptor substrate 1 and vitamin D receptor, and found no decrease in their levels (Fig. 8, which is published as supporting information on the PNAS web site). Taken together, these experiments demonstrated that siRNAs against IGF-1R, ERα, and Shc were specific and effectively down-regulated the selective target proteins in MCF-7 cells.

We next tested whether Shc is required for the physical interaction between IGF-1R and ERα. MCF-7 cells were trans-

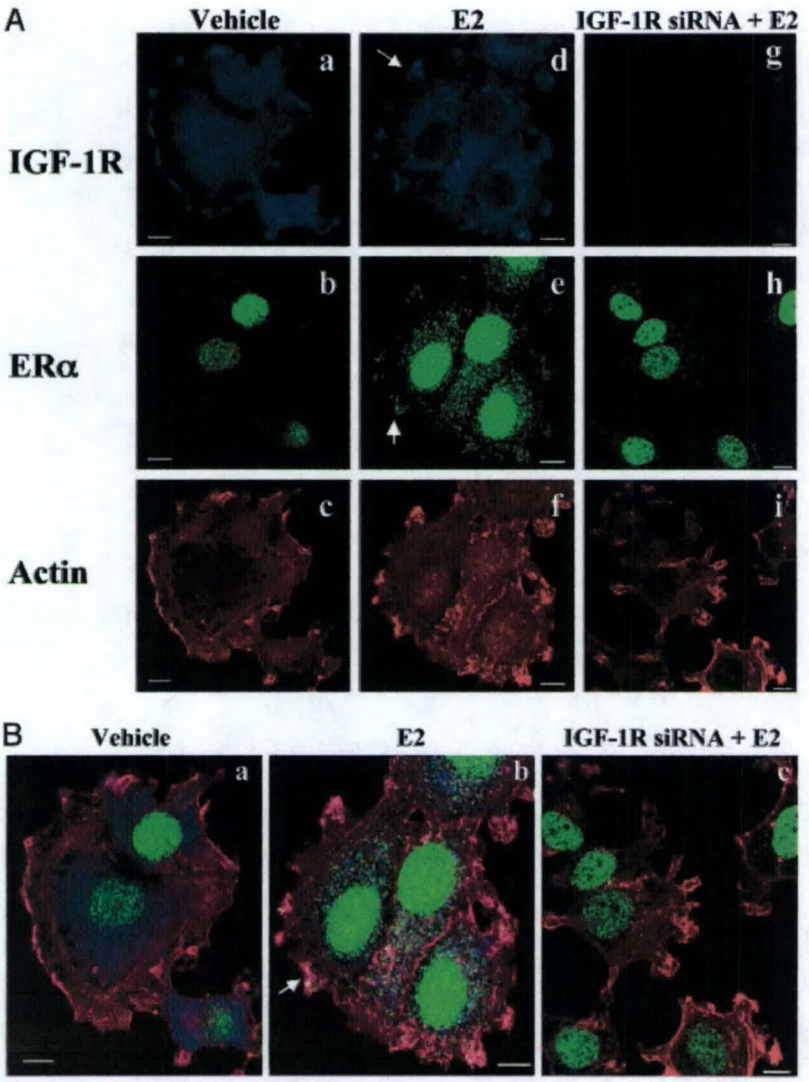

Fig. 4. IGF-1R involvement in E2-induced ERα membrane association in MCF-7 cells. Cells cultured on coverslips were transfected with or without siRNA against IGF-1R for 2 days and then treated with vehicle or E2 at 0.1 nM for 15 min. After fixation, cells were immunofluorescently stained with anti-IGF-1R (blue) anti-ERα (green) antibodies. Actin was stained with phalloidin (red), indicating filamentous cell membrane. Both nonmerged (*A*) and merged (*B*) images are shown.

fected with or without specific siRNAs against Shc for 2 days, followed by treatment with vehicle or E2. The interaction of ERα with IGF-1R was first assayed by immunoprecipitation of IGF-1R and detection of ERα on Western blot (Fig. 2*C*). Expression of the nonspecific siRNA did not block E2-induced enhancement of ERα/IGF-1R association. In marked contrast, expression of the siRNA against Shc blocked this association by 87%. To confirm this observation, a reciprocal order of antibodies was used. Fig. 2*D* shows that IGF-1R is coimmunoprecipitated with ERα, which was increased by E2 treatment in control siRNA transfected cells. Down-regulation of Shc blocked both basal level as well as E2-induced ERα/IGF-1R interaction. Together, our data demonstrated that Shc plays an important role in MCF-7 breast cancer cells by linking ERα to the membrane receptor IGF-1R.

Functional Role of Shc in Mediating the Membrane Effects of ERα. We focused on the colocalization of Shc and ERα in response to E2 in the cells with or without Shc siRNA transfection. Fig. 3*A* represents nonmerged individual stained images of Shc, ERα, and actin. Fig. 3 *Left*, *Center*, and *Right* are images of vehicle-treated cells, E2-treated cells, and cells treated by E2 after Shc down-regulation by siRNA, respectively. Under basal conditions, Shc is mainly expressed in the cytoplasm (Fig. 3*Aa*) and ERα mainly in the nucleus with minimal scattered staining in the cytoplasm (Fig. 3*Ab*). In marked contrast, E2 rapidly translocates Shc to the region of the plasma membrane as the arrow pointed (Fig. 3*Ad*). E2 appeared also to translocate ERα into the region of the plasma membrane (Fig. 3*Ae*). As further evidence of the efficacy of the siRNA against Shc to knock down this adaptor protein, the blue staining representing Shc decreased by 90% (Fig. 3*Ag*). Shc knock down strikingly abrogated the ability of E2 to translocate ERα to the region of the plasma membrane. This did not result from down-regulation of ERα itself, as evidenced by the persistence of nuclear ERα staining (Fig. 3*Ah*).

To provide further evidence of protein colocalization and cell morphologic changes in response to E2, we used a three-color merged image technique (Fig. 3*B*). Under basal conditions (Fig. 3*Ba*), cells show fewer ruffles on the membrane. Much of the Shc staining was actually cytoplasmic rather than membranous, as indicated by the blue color. ERα was seen primarily in the nucleus but also expressed in cytoplasm. E2 treatment stimulated Shc and ERα membrane region association where they colocalized along the cell membrane, as indicated by the white color development

because of the overlapping of blue/green/red colors (Fig. 3*Bb*). In many instances, ERα seemed to be collected specifically on the upper surface of extending filopodia rather than on the more basal extending edge. Expression of siRNA directed against Shc greatly reduced Shc protein expression (Fig. 3*Bc*), which also abolished ERα membrane association induced by E2. A striking finding was rapid induction of E2 induced cell morphologic changes, such as ruffles and filopodia as shown in Fig. 3*Bb*. Blockade of ERα membrane association by down-regulation of Shc did not abrogate the E2-stimulated cell morphologic changes (Fig. 3*Bc*), indicating that the morphologic changes are not regulated by the ERα/Shc membrane association. Collectively, these results demonstrated that Shc as a molecule mediates ERα rapid membrane association, and this ERα/Shc membrane association is not involved in E2-induced specific changes on cell morphology.

IGF-1R Is a Membrane Protein for Shc/ERα Binding. To test whether IGF-1R is a docking protein on the cell membrane for ERα/Shc association, a confocal microscopy experiment was performed by immunostaining of IGF-1R (blue), ERα (green), and phalloidin (blue). IGF-1R is expressed focally along the cell membrane (Fig. 4*A*) as well as in the cytoplasm (Fig. 4*Aa*). E2 treatment had little effect on IGF-1R distribution (Fig. 4*Ad*) but induced the ERα membrane translocation (arrow in Fig. 4*Ae*). Expression of IGF-1R siRNA greatly down-regulated IGF-1R protein expression in the cells (Fig. 4*Ag*), as indicated by decreased blue color. Down-regulation of IGF-1R clearly blocked ERα membrane translocation in the cells (Fig. 4 *Ah* and *Bc*), confirming the role of IGF-1R on ERα membrane association. E2 rapidly induced cell motile structure formation (Fig. 4*Ab*), compared with vehicle treatment (Fig. 4*Ba*). At the same time, down-regulation of IGF-1R had little effect on the E2-induced cell morphologic changes (Fig. 4*Bc*). Together, our data demonstrated that both Shc and IGF-1R are involved in ERα membrane association in MCF-7 cells but not in E2 action on cell motile structure development.

ERα Membrane Association Is Directly Correlated to E2-Induced MAPK Phosphorylation. We examined the effect of E2 on MAPK phosphorylation in cells in which Shc, ERα, or IGF-1R was selectively knocked down. The rapid induction of MAPK activation served as the biologic endpoint reflecting functionality. As shown in Fig. 5*A*, treatment with physiologic concentrations of E2 at 0.1 nM resulted in a 3.4-fold increase of MAPK phosphorylation in control siRNA transfected cells. The siRNAs against IGF-1R, ERα, or Shc specifically blocked E2-induced MAPK phosphorylation, indicating the involvement of IGF-1R, Shc, and ERα in E2 action on MAPK. Elevated basal MAPK phosphorylation was seen in the cells expressing siRNA against IGF-1R. To further confirm the involvement of IGF-1R on E2-induced MAPK activation, a specific IGF-1R tyrosine kinase inhibitor AG1024 was tested along with other inhibitors. Fig. 5*B* shows that E2 greatly increased MAPK phosphorylation, which was blocked by AG1024, ICI, and PP2, indicating that IGF-1R, ERα, and c-Src are all upstream components regulating MAPK in E2 rapid action. AG 1024 also blocked cell proliferation as biologic evidence for the role of IGF-1R in E2-induced cell proliferation (Fig. 9, which is published as supporting information on the PNAS web site).

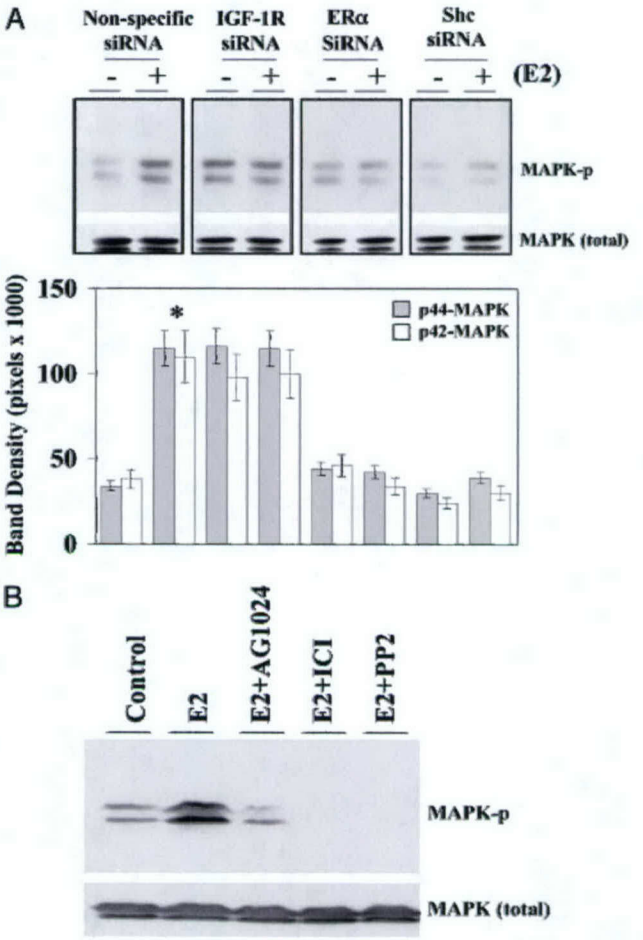

Fig. 5. Evidence of the involvement of ERα, Shc, and IGF-1R in E2-induced MAPK phosphorylation. (*A*) Down-regulation of ERα, Shc, and IGF-1R blocked E2-induced MAPK phosphorylation. Cells were transfected with specific and nonspecific siRNAs. At day 2, cells were treated with vehicle or E2 at 0.1 nM for 15 min, and then whole-cell extracts were prepared and analyzed by Western blotting as described in *Materials and Methods*. Polyvinylidene difluoride membranes were probed with antidual phosphotyrosine MAPK antibody. *, $P < 0.05$ comparison of E2-treated cells with the vehicle-treated control in each siRNA transfected group. (*B*) Selective pathway inhibitors on E2-induced MAPK phosphorylation. Cells were challenged with vehicle or 0.1 nM E2 for 15 min in the presence of the inhibitors, as indicated.

Discussion

ERα mediates the effects of E2 at the level of the nucleus through classic genomic actions on transcription and additionally initiates rapid nongenomic events via membrane-associated interactions. The present study examined the mechanism by which the ERα can translocate to the region on or near the plasma membrane. We postulated that the adaptor protein Shc serves as the translocator by binding to ERα and then carrying this receptor to Shc-binding sites of IGF-1R located on the cell membrane. Thus ERα translocates to the plasma membrane region via the same mechanisms that allow Shc to locate there. Our previous studies demonstrated that E2 induced direct interaction of ERα with Shc and caused translocation of ERα to the region of the plasma membrane (9). Our current findings provide several lines of evidence to support our hypothesis regarding ERα/Shc/IGF-1R interactions leading to ERα membrane association. We demonstrated that Shc and IGF-1R are strictly required for translocation of ERα to the membrane region and for E2 induction of MAPK activation. Immunoprecipitation and confocal localization studies demonstrated that E2 causes the formation of ternary protein complexes involving Shc, ERα, and the IGF-1R. More importantly, selective knock down of Shc or IGF-1R with siRNA technology or use of the antiestrogen, ICI, disrupts the ternary complexes. Knock down of Shc abrogates the ability of E2 to translocate ERα to the membrane region and to activate MAPK. As evidence of biologic function, an inhibitor of IGF-1R phosphorylation blocks E2-induced cell proliferation (Fig. 9). Taken together, our findings demonstrate a previously undescribed function of Shc that links ERα to the region of the cell membrane by binding to IFG-1R.

Members of the steroid hormone family classically act through transcriptional regulation at the nuclear level (18). However, increasing evidence suggests that all of these hormones also exert rapid membrane-mediated effects on their target cells (2, 19–22). Controversy exists regarding the identity of the membrane receptor proteins and specifically whether they are classical receptors or other binding proteins (6, 8, 9) Down-regulation of ERα by the selective siRNA in the present study clearly demonstrates the role of ERα itself in mediating the effects of E2 on MAPK activation. Li *et al.* (23) have described a role for truncated forms of ERα on the membrane, as reviewed in detail.

Transmembrane receptors for growth factors, such as IGF-1 and epidermal growth factor receptor (EGFR), are believed to be integral components in the growth response to E2, but the specific steps are unclear. The tyrosine phosphorylation and activation of IGF-1R induced by E2 were reported in uterine epithelial cells and ER-transfected COS-7 cells (24–26). Previously, we demonstrated strong interaction between ERα and Shc through N-terminal ERα and PTB/SH2 domains of Shc in MCF-7 cells (9). Shc is a well-defined molecule, which mediates growth factor mitogenic actions by activation of the MAPK pathway (27). Activation of IGF-1R will recruit Shc binding on the receptors (28). Regarding IGF-1R, it is noteworthy that a large amount of evidence pointed out that Y950 of IGF-1R is a major site for Shc binding, phosphorylation, and activation in response to IGF-1 (16, 29). IGF-1R functions as a membrane-docking site that recruits the adapter protein Shc after the receptor is activated. Nevertheless, more complicated mechanisms exist for the involvement of IGF-1R, because phosphorylated IGF-1R also recruits the p85 regulatory domain of phosphatidylinositol 3-kinase binding to the receptor. This domain has been reported to physically associate with ERα in human vascular endothelial cells and MCF-7 cells (30, 31). This might explain our biochemical findings that down-regulation of Shc decreased the level of ERα associated with IGF-1R but did not completely eliminate ERα and IGF-1R interaction.

EGFR, in a fashion analogous to IGF-1R, is also a transmembrane tyrosine kinase receptor that has been engaged in different processes critical for cell proliferation, survival, and tumor invasion. E2 has been reported to activate MAPK by activation of matrix metalloproteinases-2 and -9, heparin-binding EGF release, and activation of EGFR (7, 32). The interaction between the signaling pathways of ERα and the EGFR is known to contribute to the biological effects of E2. Experiments show that EGF antibody prevents E_2-induced vaginal and uterine growth (33), implying that crosstalk from ERα to the EGFR at the membrane may be physiologically important. The EGFR signaling pathway is important in the development of reproductive tissues (34, 35). The requirement for EGFR on E2-induced ERα-membrane association is currently under investigation in our laboratory.

The present study demonstrated a dramatic dissociation between the requirement of Shc for MAPK activation and for dynamic membrane changes. Specifically, knock down of Shc blocked MAPK activation by E2 but not dynamic membrane changes (Figs. 3*Bc* and 4*Bc*). Our working hypothesis to explain this dichotomy of effects is that an ERα/Shc/IGF-1R complex in the plasma membrane may be needed to activate MAPK. On the other hand, the presence of ERα in the cytoplasm contiguous with dynamic membrane changes may be sufficient to induce dynamic membrane changes. Based on our findings, we propose a model for E2-induced ERα membrane association and its function on MAPK activation (Fig. 10, which is published as supporting information on the PNAS web site). In this model, liganded ERα interacts with Shc, leading to Src activation; Src in turn phosphorylates IGF-1R, which recruits Shc; and Shc acts as a translocator to bring ERα to the cytoplasmic membrane. Association of Shc with membrane IGF-1R serves two functions; one is to lead to ERα membrane association, and the other is to activate MAPK in IGF-1R-dependent manner. An extensive discussion is available as supporting information on the PNAS web site.

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