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PRINCIPAL INVESTIGATOR: Ellis R. Levin, M.D.

CONTRACTING ORGANIZATION: The University of California, Irvine
Irvine, California 92697

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13. ABSTRACT (Maximum 200 Words) We investigated the mechanisms of membrane estrogen receptor signaling to breast cancer. We discovered that serine 522 of mouse ER α is required for membrane localization. Importantly, when expressed in MCF-7 cells, this S522A mutant ER α heterodimerizes with endogenous ER α , sequestering it from the membrane and inhibiting ERK activation by E ₂ . This mutant has no effect on endogenous nuclear ER. We found that cyclin D ₁ synthesis, Cdk4 activity and G ₁ /S progression is stimulated via ERK and inhibited by S522A mutant ER α expression in MCF-7 (Mol Cell Biol 23(3):1633-46, 2003). We also found that estradiol signaling to ERK involves transactivation of the EGF receptor, through G _q α and G _i α signaling to Src, Mmp activation and HB-EGF liberation. Only the E domain of ER α is required for these events initiated at the plasma membrane (J Biol Chem 278:2701-12, 2003).				
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Introduction

Estrogen promotes an increased incidence of breast cancer in women, while anti-estrogen therapy both limits recurrences and prevents the development of primary disease in genetically predisposed individuals. The purpose of the studies proposed in our grant is to determine the cellular mechanisms by which estrogen enhances the survival of breast cancer.

Body

In conjunction with the statement of work, we continue to determine mechanisms by which estradiol (E_2) and membrane estrogen receptors (ER) act as survival and growth stimulus for breast cancer. We found that serine 522 of mouse ER α is necessary for plasma membrane localization. When expressed in MCF-7 or 2R-75-1 cells, a mutant S522A ER α heterodimerizes and sequesters the membrane ER but not the nuclear ER, leading to ERK downregulation (but no effect on ER E-transcription). We showed that E_2 -induced ERK importantly contributed to cyclin D₁ synthesis, increased Cdk4 activity against the retinoblastoma protein (inactivating phosphorylation), and subsequent G₁/S cell cycle passage. This was all markedly inhibited by expression of the S522A mutant ER α in the breast cancer cells. The data mechanistically support a role for ERK and membrane ER in mediating breast cancer proliferation and survival, and validate a novel reagent to specify membrane-initiated versus nuclear-initiated signaling by estrogen in breast cancer (Mol Cell Biol 23:1633-46, 2003).

We also determined the mechanism by which membrane ER signals to ERK in breast cancer. This involves a cross talk to the EGF receptor, which is transactivated by the membrane and not nuclear ER. In fact, it is the E domain of ER α that is sufficient to accomplish this. E_2 ligation of membrane ER α causes a G_q α and G₁₂ α -dependent activation of Src via PKC and calcium signaling. This leads to Src-induced matrix metalloproteinase 2 and 9 cleavage/activation and liberation of Hb-EGF. Hb-EGF then binds and activates EFR, leading to both ERK and PI₃K upregulation in MCF-7 cells (J Biol Chem 278:2701-12, 2003). This was also reviewed in Mol Endo 17:309-17, 2003.

We also determined that signaling from the membrane receptor through PI₃ kinase resulted in the upregulation of 250 genes in the endothelial cell. This indicates that referable to the tumor vasculature, transcription of important target genes that participate in angiogenesis and other processes is modulated by the membrane ER signaling (J Biol Chem 277:50768-75, 2002).

Very recently, we addressed the issue of whether intact BRCA1 interacts with membrane ER function to prevent signaling to cell proliferation and survival. In initial studies, we found that in MCF-7 and 2R-75-1 cells, that expression of intact BRCA1 inhibits E_2 -induced ERK and cell proliferation over a 72-hour period. In contrast, three mutant BRCA1 proteins found in women with breast cancer do not affect these functions of ER. Intact BRCA1 also inhibited EGF-induced ERK and cell proliferation. Our preliminary studies suggest that BRCA1, in the setting of E_2 , induces a MAP kinase phosphatase, MKP-1 and enhances phosphatase activity to

downregulate ERK. We intend to further investigate these interactions and their impact for breast cancer.

Key Research Accomplishments

- Identification of a structural determinant (serine 522) that is necessary for membrane localization of ER α .
- Development of a new specific reagent, a mutant ER α (S522A) that only downregulates endogenous membrane ER function.
- Proving that signaling through ERK by membrane ER is important for cell cycle progression of breast cancer.
- Elucidation of the important cross talk steps between membrane ER and the EGF receptor, resulting in breast cancer proliferation.

Reportable Outcomes

Abstracts and Presentations

1. Razandi M, Alton G, Pedram A, Shonshani S, Levin ER. Serine 522 of mouse estrogen receptor alpha is essential for membrane localization, signaling and cell biology. Presented at the 84th Annual Meeting of the Endocrine Society, San Francisco, CA, June 2002.
2. Pedram A, Razandi M, Park ST, Levin ER. Proximal events in membrane estrogen receptor signaling requires G-protein induced transactivation of the EGF receptor. Presented at the 84th Annual Meeting of the Endocrine Society, San Francisco, CA, June 2002.

Manuscripts

1. Pedram, A., Razandi, M., Aitkenhead, M, Hughes, CCW, and Levin, ER. Integration of the non-genomic and genomic actions of estrogen: membrane initiated signaling by steroid (MISS) to transcription and cell biology. J Biol Chem 277(52):50768-50775, 2002.
2. Razandi M, Pedram, A, Parks S, and Levin ER. Proximal events in ER signaling from the plasma membrane. J Biol Chem 278:2701-2712, 2003.
3. Levin, ER. Bi-directional signaling between the estrogen receptor and the epidermal growth factor receptor. Mol Endocrinol 17:309-17, 2003.
4. Razandi, M, Alton, G, Pedram, A, Ghonshani S, Webb D, and Levin, ER. Identification of a structural determinant for the membrane localization of ER α . Mol Cell Biol 23(3): 1633-1646, 2003.46, 2003.

Conclusions

Estradiol signaling from membrane ER is important to breast cancer cell survival and growth. The ERK pathway is very important in this regard, and activation of ERK by membrane ER requires cross talk to the EGF receptor. These results justify developing ER antagonists that selectively act at the cell membrane, to help prevent and treat breast cancer.

References

None.

Appendices

Four articles attached.

Revised Statement of Work

"Novel mechanisms by which estrogen induces anti-apoptosis in breast cancer"

Aim 1 - To determine the important cellular interactions between intact and mutant BRCA1 and membrane ER that inhibit estrogen-induced proliferation and survival of breast cancer. These studies use MCF-7 and HCC-1937 cells (07/03-07/04).

- a.** Show that intact but not mutant(s) BRCA1 block estrogen-induced signaling through ERK to breast cancer cell proliferation and survival, via membrane ER (months 1-6)
- b.** Demonstrate that intact BRCA1 induces a MAP kinase phosphatase (MKP-1) that inhibits ERK activation by estradiol. This occurs at the level of transcription and enzyme activation (months 1-6).
- c.** Show that intact BRCA1-1 also blocks EGF and IGF-1 induced ERK signaling to proliferation (months 6-12).
- d.** Determine the specific cell cycle events that are activated by membrane ER signaling via ERK, and that are inhibited by E_2 . This includes cyclin D₁, Cdk4, cyclin B₁ and Cdc2 induction and activity (months 6-12).

Integration of the Non-genomic and Genomic Actions of Estrogen

MEMBRANE-INITIATED SIGNALING BY STEROID TO TRANSCRIPTION AND CELL BIOLOGY*

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Ali Pedram[‡], Mahnaz Razandi[‡], Mark Aitkenhead^{‡¶}, Christopher C. W. Hughes^{‡¶},
and Ellis R. Levin^{‡¶**}*From the [‡]Division of Endocrinology, Veterans Affairs Medical Center, Long Beach, Long Beach, California 90822 and the Departments of [§]Medicine, [¶]Pharmacology, and ^{¶¶}Molecular Biology and Biochemistry, University of California, Irvine, Irvine, California 92717*

Estrogen binds to receptors that translocate to the plasma membrane and to the nucleus. The rapid, non-genomic actions of this sex steroid are attributed to membrane action, while gene transcription occurs through nuclear receptor function. However, gene transcription can also result from estrogen signaling initiated at the membrane, but the relative importance of this mechanism is not known. In vascular endothelial cells (EC), estradiol (E_2) activates several kinase cascades, including phosphatidylinositol 3-phosphate (PI3K)/Akt, a signaling pathway that impacts EC biology. We determined here by DNA microarray that 40-min exposure to E_2 significantly increased 250 genes in EC, up-regulation that was substantially prevented by the PI3K inhibitor, LY294002. This coincided with maximum E_2 -induced PI3K activity at 15–30 min. An important vascular gene strongly up-regulated by E_2 in our array produces cyclooxygenase-2 (Cox-2). In cultured EC, E_2 induced both Cox-2 gene expression and new Cox-2 protein synthesis by 40 and 60 min, respectively, and rapidly stimulated the secretion of prostaglandins PGL_2 and PGE_2 . The up-regulation of gene expression reflected transcriptional transactivation, shown using Cox-2 promoter/luciferase reporters in the EC. Soluble inhibitors or dominant negative constructs for PI3K and Akt prevented all these actions of E_2 . Functionally, EC migration was induced by the sex steroid, and this was significantly reversed by NS-398, a Cox-2 inhibitor. Gene transcription and cell biological effects of estrogen emanate from rapid and specific signaling, integrating cell surface and nuclear actions of this steroid.

Estradiol (E_2)¹ and other steroid hormones are traditionally considered to transactivate target genes after binding nuclear

receptors (1). However, E_2 also has rapid, non-genomic effects (2–4), and these have recently been attributed to cell membrane-initiated signaling. At the cell surface, a small population of ER binds E_2 and activates G proteins (5–7). Multiple signaling pathways are then rapidly stimulated by E_2 in target cells that express endogenous ER α and ER β , and these pathways have been linked to discrete cellular actions of the steroid (8–11). In this respect, a truncated MTA1 protein was recently found to be highly expressed in aggressive breast cancer (12). This protein sequesters ER away from the nucleus and strongly reduces E_2 -activated transcription yet promotes increased ERK signaling and aggressive behavior of the tumor. It is proposed therefore that the integration of cell surface and nuclear signaling impacts overall cell biology (13).

Signaling from the membrane leads to the post-translational modification of important structural and functional proteins in the cell. In EC, E_2 activates the p38-MAPKAP-2 kinase pathway; MAPKAP-2 phosphorylates and modifies the function of heat shock protein 27 (14). This important modification leads to the morphological preservation and survival of the EC and stimulates primitive capillary tube formation. In breast cancer, E_2 acts as a cell survival factor, in part by preventing chemotherapy or radiation-induced JNK activation (9). JNK phosphorylates and inactivates Bcl2 and Bcl-xl, leading to the assembly of the apoptosome and caspase-mediated cell death. By preventing JNK activation and Bcl2/Bcl-xl phosphorylation, E_2 rescues the breast cancer cells (9). This provides a mechanism for the ability of E_2 to oppose therapeutic interventions in this malignancy.

In addition to post-translational protein effects, E_2 is recognized to stimulate transcription through signaling typically initiated at the membrane. As precedent for this effect of E_2 , growth factor tyrosine kinase receptors (insulin-like growth factor-1 receptor and epidermal growth factor receptor) exist in the plasma membrane and signal through common kinase cascades to gene transcription. E_2 effects may involve the G protein-initiated, signaling-induced synthesis or activation of transcription factors. E_2 stimulates c-fos through ERK- or PI3K-dependent pathways (15–17), the BCL-2 gene through the modification of Sp-1 transcription factor (18), and the prolactin gene through ERK (19). E_2 activation of PI3K in EC results from the membrane ER-p85 regulatory subunit (PI3K) association, and this signaling to nitric oxide production rescues rats from ischemia-reperfusion injury (20). However, insight is largely lacking as to the full extent and importance of the specific integration of membrane signaling and nuclear effects of the sex steroid.

We therefore identified the transcriptional targets in EC that are rapidly up-regulated from E_2 signaling through the activation of PI3K. We also describe in depth the ability of

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** To whom correspondence should be addressed: Medical Service (111-I), Long Beach Veterans Administration Medical Center/UC-Irvine, 5901 E. 7th St., Long Beach, CA 90822. Tel.: 562-826-5748; Fax: 562-826-5515; E-mail: ellis.levin@med.va.gov.

¹ The abbreviations used are: E_2 , estradiol; Cox, cyclooxygenase; EC, endothelial cells; ER, estrogen receptor; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; MAPKAP, mitogen-activated protein kinase activated protein kinase; PI3K, phosphatidylinositol 3-phosphate; RT, reverse transcriptase; PGL_2 , prostaglandin I_2 ; PGE_2 , prostaglandin E_2 ; NO, nitric oxide; EGR, early growth response; bFGF, basic fibroblast growth factor; DN, dominant negative; MISS, membrane-initiated steroid signaling; PTTG, pituitary tumor transforming gene; BAEC, bovine aortic endothelial cell.

E_2 -induced PI3K activation to up-regulate one specific gene, *Cox-2*, leading to the production of the enzyme, secretion of products of *Cox-2* activation, and EC migration. This approach can be used to identify programs of gene activation that result from membrane-initiated steroid signaling (MISS)² by E_2 .

MATERIALS AND METHODS

PI3K Activity Assay—EC were incubated with/without 10 nM E_2 and 10 μ M LY294002 for up to 6 h. Cells were then lysed, and the lysates pelleted then dissolved in SDS sample buffer, boiled, separated, and transferred onto nitrocellulose. Phosphorylated Akt was detected using phospho-specific monoclonal antibodies (Santa Cruz Biotechnology) and the ECL Western blot kit. Equal samples from the lysates were also immunoprecipitated, and immunoblots of the precipitated kinase protein from each experimental condition were determined to show equal gel loading. All experiments were repeated two or three times.

DNA Arrays—Human umbilical vein endothelial cells were incubated without or with 10 nM E_2 with or without the specific PI3K inhibitor, LY294002 (10 μ M), or LY294002 alone, for 40 min. For validation, the experiment was repeated a second time. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and by RNeasy columns (Qiagen, Valencia, CA). Total RNA was adjusted to 1 μ g/ μ l, and first-strand cDNA, followed by double-stranded cDNA was synthesized from poly(A)⁺ mRNA by the Microarray Facility at the University of California, Irvine. This was done using the SuperScript double-stranded cDNA synthesis kit (Invitrogen) and poly(T) nucleotide primers that contained a sequence recognized by T7 RNA polymerase. A portion of the resulting double-stranded cDNA was used as a template to generate biotin-tagged cRNA from an *in vitro* transcription reaction (IVT) using the BioArray High-Yield RNA transcript labeling kit (T7) (Enzo Diagnostics, Farmingdale, NY). 15 μ g of the biotin-tagged cRNA was fragmented to strands of 35–200 bases in length following prescribed protocols (Affymetrix GeneChip Expression Analysis Technical Manual). 10 μ g of the fragmented cRNA was hybridized with rotation at 45 °C for 16 h to probe sets present on an Affymetrix human U95a array (Affymetrix, Santa Clara, CA). The arrays were automatically washed and stained with streptavidin-phycoerythrin. Probe arrays were then scanned on a Hewlett-Packard GeneArray scanner. Affymetrix Microarray Suite 5.0 was used to quantify and analyze the average difference in intensities between conditions of the experiment, for each represented gene. The comparisons between conditions were outputted as -fold increase or decrease, or no change, and data were compared with determine which genes were E_2 -responsive in a PI3K-dependent fashion. This output was then inserted into a stringent, Bayesian-based statistical analysis program (Cyber-T) (21), available as a web interface at the University of California at Irvine. The upper 2.5% of genes identified as being significantly different by Cyber-T (and in agreement with the Affymetrix analysis) are presented in table form.

RT-PCR and Reporter Assays—Validation of differential expression was performed by RT-PCR for four genes identified in the microarray and was standardized to glyceraldehyde-3-phosphate dehydrogenase. cDNA was prepared from 3 μ g of total RNA isolated from EC, primed with random hexamers (Invitrogen), and reverse-transcribed with Superscript II (Invitrogen) per the manufacturer's instructions. The primers were prepared by Invitrogen for the following sequences; *Cox-2*, TGGGAAGCCTTCTAACCCTCTCCT and CTTTGACTGTGGGAGGATACATCTC; *CREM*, TGGAAACAGTTGAATCACAG; and CTACTAATCTGTGTTTGGGAG; *EGR2*, CAGTACCCTGGTGCCAGCTG and TGTGGATCTCTCTGGCACGG; *JUN-B*, CCGGATGTGCACGAAAATGGAACAG and ACCGTCCGCAAAGCCCTCCTG; glyceraldehyde-3-phosphate dehydrogenase; ACCACAGTCCATGCCATCAC and TCCACCACCTGTGCTGTA. PCR reactions were performed using 200 nM of primers with 2 μ l of cDNA in 50 μ l of Platinum PCR supermix (Invitrogen). After an initial denaturation step of 94 °C for 4 min, 25–35 cycles of 94 °C for 30 s, 55–61 °C for 45 s, 72 °C for 30–45 s, and a final extension at 72 °C for 10 min. The PCR products were separated by electrophoresis in a 1.2% agarose gel and visualized by ethidium bromide staining. The cycle number and annealing temperature was adjusted for each of the genes amplified.

For reporter assays, BAEC were transiently transfected with 10 μ g of the PGL-3 plasmid containing 1.8 kb of the human *Cox-2* promoter driving a luciferase reporter fusion protein (22), kindly provided by Dr.

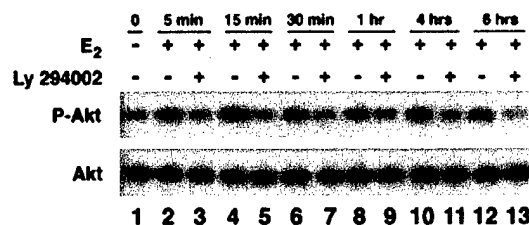


FIG. 1. Time course of PI3K activation in endothelial cells by estradiol (E_2). Cells were incubated with 10 nM E_2 with or without 10 μ M LY294002 for the times indicated, and Akt phosphorylation as a function of PI3K activity was determined by Western blot. Immunoblot of total Akt protein is shown below each condition. A representative study of two completed is shown.

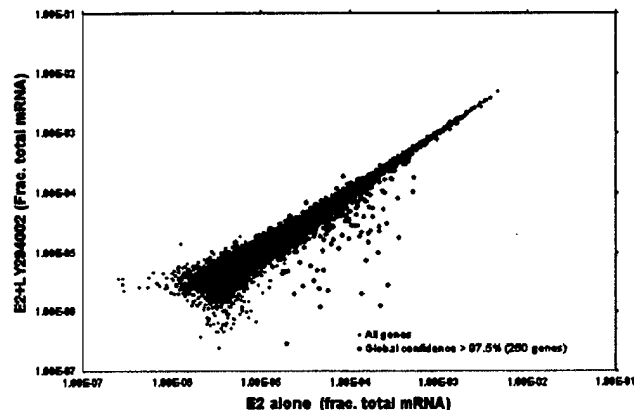


FIG. 2. Estrogen-induced genes in endothelial cells, dependent upon phosphatidylinositol 3-kinase signaling. EC were incubated/not incubated with 10 nM E_2 with or without LY294002 (PI3K inhibitor), or LY294002 alone, for 40 min. Total RNA from the four conditions was extracted for purposes of analysis after cDNA/cRNA synthesis (see "Materials and Methods"). The figure is the output from CyberT analysis, a stringent, Bayesian-based statistical analysis program. Duplicate determinations for all conditions were utilized for the analysis. Genes in red are the upper 2.5% of all genes positively regulated by E_2 and mostly inhibited by LY294002.

David Dixon (Vanderbilt). The liposome-mediated transfection was carried out as previously described (6, 9), and the cells were recovered in serum overnight, then synchronized without serum over 24 h before experiments (in phenol red-free medium). Cells were then incubated for 6 h with 10 nM E_2 with or without either LY294002 (10 μ M), a soluble inhibitor of NF κ B (SN50M), or the corresponding inactive control (SN50), each at 20 μ M. All inhibitors were from Calbiochem (San Diego, CA). Assays were quantified by luminometer, and the results were adjusted for expression of co-transfected *Renilla* luciferase. Triplicate determinations per condition were carried out in each of two experiments. The combined data were analyzed by analysis of variance plus Scheffé's test, at a $p < 0.05$ level of significance.

***Cox-2* Protein and PGI₂ Secretion**—For protein synthesis, BAECs were exposed to 0.1–10 nM E_2 for 60 min, preceded where indicated for 30 min by incubation with ICI 182,780 (1 μ M), LY294002 (10 μ M), or wortmannin (PI3K inhibitor) (100 nM). Additional BAECs were either transiently transfected with dominant negative myc-tagged pMT2-AH-Akt (kindly provided by Dr. Julian Downward) (23), or dominant negative PI-3K p85 subunit (pcDNA3-delta p85, lacking residues 478–513) (kindly provided by Dr. Barry Posner) (24), as previously described (25), or with pcDNA3 as control. The cells were recovered and synchronized over 24 h then incubated with E_2 .

For PGI₂ (measured as 6-keto-PGF1 α) and PGE₂ secretion studies, BAEC were incubated for 0–240 min with E_2 , to determine time of peak secretion. This was found to be 30 min, as subsequent time points reflected both secretion and accumulation. Additional studies were then conducted at 30 min. BAEC were incubated with/without 10 nM E_2 , with or without inhibitors, including NS-398 (5 μ M) (*Cox-2* inhibitor), or SC-560 (20 nM) (*Cox-1* inhibitor). Each condition was run in duplicate. The incubation media were collected and concentrated by lyophilization, and prostaglandin concentrations were determined by enzyme-

² Terminology to replace "non-genomic" effects of steroids, as suggested by the Consensus Working Group at the FASEB conference on Membrane Steroid Receptors, Aspen, CO, June 22–27, 2002.

TABLE I
Genes significantly up-regulated by estradiol in a PI3K-dependent fashion

Genes that were induced at least 2-fold in endothelial cells after 40-min exposure to E_2 but were not significantly induced after exposure to E_2 and LY294002 are listed. Differentially expressed genes were identified by both Affymetrix analysis software and the Cyber T (Bayesian) statistical analysis program.

Description	Accession #	Fold Increase	Description	Accession #	Fold Increase		
Transcription Factors			Signaling				
early growth response-3 protein	EGR-3	X63741	70.49	Cot/Tpl-2 kinase	MAP3K	D14497	13.64
early growth response 2 protein	EGR-2	J04076	66.84	GTP-ase	gem	U10550	11.34
Zinc finger protein 36	GOS24	M92843	20.25	protein-tyrosine phosphatase	CL 100	X68277	11.07
Arb6 Transcription factor	AREB6	D15050	18.47	corticotrophin releasing factor receptor	CRHR1	X72304	7.97
early growth response 1 protein	EGR 1	S81439	10.10	putative topoisomerase-III	TOPO3	D87012	6.46
DEC-1 transcription factor	BHLHB2	AB004066	8.64	insulin receptor substrate-1	IRS-1	S62539	6.22
zinc finger protein 137	ZFP137	U09414	7.02	STAT-induced STAT inhibitor-2	STAT2	AF037989	5.55
Max dimerization protein	MAD	L06895	7.01	protein tyrosine phosphatase	PTP-U2	Z48541	4.94
myogenic factor 5	Myf-5	X14894	6.35	Sprouty 2	SPRY2	AF039843	4.58
jun B proto-oncogene	JunB	X51345	6.24	Orphan G protein-coupled receptor	RDC1	U67784	4.55
activating transcription factor 3	ATF3	L19871	6.01	SRC-like tyrosine kinase	FRK	U00803	4.46
CREM-beta;	CREM	S68134	5.73	glycerol kinase	GKTB	X78711	4.10
c-fos oncogene	c-fos	V01512	5.05	Toll like receptor 2	TIL4	AF051152	3.83
zinc finger protein	FDZF2	U95044	4.67	serine/threonine protein kinase	sgk	Y10032	3.71
msg-related gene 1	msg1	U65093	4.55	G protein-coupled receptor	CNR1	U73304	3.59
immediate early protein	ETR11	M62831	4.34	Wnt-5a	Wnt-5a	L20861	3.51
Forkhead box C1	FKHL7	AF078096	4.18	PI3K regulatory 1	GRB1	M61906	3.01
B Cell lymphoma protein 6	bcl-6	U00115	4.16	GS3955	GS3955	D87119	2.97
c-jun oncogene	c-JUN	J04111	3.99	Wnt-7a	WNT7a	D83175	2.87
c-myc oncogene	c-myc	V00568	3.86	chapsyn-110	DLG2	U32376	2.83
DNA damage inducible transcript 3	DDIT3	S62138	3.76	neurotrophic tyrosine kinase receptor 3	trkC	S76475	2.81
immediate-early gene X1	IEX-1	S81914	2.93	serum-inducible kinase	SNK	AF059617	2.68
special AT-rich sequence binding protein 1	SATB1	M97287	2.91	P2Y6 receptor	P2Y6R	AF007893	2.67
butyrate response factor 2 (EGF-response factor 2)	ERF-2	X78992	2.77	intra-acrosomal protein	SP-10	S65583	2.63
Inhibitor of growth like-1	ING1L	AB012853	2.68	SA (rat hypertension-associated) homolog	SA	X80062	2.61
KRAB zinc finger	ZNF75	S67970	2.66	type II interleukin-1 receptor	IL-1R2	X59770	2.58
Regulator of G protein signaling 2	RGS2	L13463	2.44	Dual Specificity protein phosphatase 6	DUSP6	AB013382	2.54
Hairy enhancer of split1	HES1	L19314	2.40	guanine nucleotide exchange factor	SOS1	L13857	2.52
basic helix-loop-helix transcription factor 15	TCF15	U08336	2.35	Nuclear Factor of Activated T cells x4	NFATx4	U85430	2.38
basic helix-loop-helix transcription factor 2	TCF2	U19345	2.30	plasma membrane calcium ATPase	PMCA2	X63575	2.38
Sprouty 1	spry-1	AF041037	2.30	SNF1-like protein kinase	SNFLPK	U57452	2.36
positive elongation transcription factor b	P-FEBb	AF048732	2.29	MAP kinase phosphatase	MKP-2	U48807	2.33
Macrophage stimulating pseudogene 9	MSTP9	U28055	2.23	inositol polyphosphate 4-phosphatase type II-alpha	IPP4P2A	U96922	2.31
IkappaB alpha	IKBA	M69043	2.23	multiple PDZ domain protein	MUPP1	AF093419	2.31
Zinc finger protein 17	ZNF17	AF041259	2.22	Chloride channel protein	CLCN3	X78520	2.19
Multi drug resistance	MDR1	X58723	2.18	putative g protein-coupled receptor	TM7SF1	AF027826	2.18
hox 5.1 protein	HSX5.1	X17360	2.11	G protein-linked receptor	GPCR	L42324	2.16
alpha-Pu1	NRF1	U02683	2.10	kappa opioid receptor	OPRK1	L37362	2.12
Sp4 transcription factor	SPR-1	X68561	2.01	thrombomodulin precursor	THBD	J02973	2.02
myocyte-specific enhancer factor 2A	MEF2A	U49020	2.00	receptor phosphatase PCP-2	PCP-2	X97198	2.02
Mesenchyme Fork Head-1	MFH-1	Y08223	2.00				

linked immunosorbent assay (Cayman Chemical). The study was repeated three times, with the results reflected in the bar graph.

EC Migration Studies—EC were grown to monolayer confluence on six-well plates and synchronized for 24 h in the absence of serum. A "wound" was created by scraping the monolayer with a single-edge razor blade, and cells were removed to the left of the wound. Serum-free Dulbecco's modified Eagle's medium containing 10 nM E_2 with or without NS-398, SC-560, LY294002, wortmannin, or alone as control was added to separate dishes of wounded EC for 24 h at 37 °C. The cells were then fixed in 3.7% formaldehyde and assessed for migration (14). BAEC migration was measured using an image analyzer system composed of an inverted microscope and a 20- to 24-inch digitizing board (Jandel Scientific, Corte Madera, CA) attached to a computer. The Sigma Scan program (Jandel) was used for analysis of measurements of the distance traveled by the cells within the calibrated area adjacent to the wound. Five measurements in each well were taken, and results from three separate experiments contributed to create the bar graph.

RESULTS AND DISCUSSION

Estrogen Stimulates PI3K Activity in EC— E_2 induced substantial Akt phosphorylation by 5 min, reflecting PI3K activation, because the phosphorylation was totally prevented by a PI3K inhibitor (Fig. 1). The peak activity occurred at 15 min and lasted for the 6-h duration of the experiment. We therefore carried out our array studies to assess genes rapidly induced by E_2 via PI3K signaling, based upon these results.

E_2 Rapidly Induces Many Genes via PI3K Activation—EC were incubated with 10 nM E_2 or without steroid (control) for 40 min, in the presence or absence of LY294002, a PI3K inhibitor.

cRNA from each experimental condition was used for microarray gene analysis, as delineated under "Materials and Methods." The DNA array hybridization pattern was analyzed by both Affymetrix statistical software and the CyberT Bayesian-based program, and 250 genes were identified as being significantly up-regulated by E_2 (upper 2.5% of all genes) (Fig. 2). This occurred in both E_2 -inducible and PI3K-reversible fashion. In comparing control cRNA (no treatment) to cRNA from cells treated with LY294002 alone, no differences were detected. We list the genes that were 1) up-regulated in this fashion by more than 2-fold and 2) identified to have some known function (Table I). In contrast, few genes were down-regulated by E_2 in the quiescent EC, and none depended upon PI3K/Akt activation, whereas several genes were stimulated by E_2 and further enhanced upon PI3K inhibition. Genes discussed in the text are given in boldface in Table I.

As might be predicted from the time chosen, many transcription factors were rapidly up-regulated. These included the *fos*, *myc*, and *jun* genes previously known to be stimulated by estrogen (15, 26, 27), which thereby validate our results. Here we extend the findings and implicate PI3K action in proto-oncogene up-regulation by E_2 . This sex steroid was recently shown to activate *c-fos* transcription in MCF-7 cells, via a PI3K/Akt pathway, targeting the serum response factor motif in the proximal *fos* promoter (16). We also found stimulation of many transcription factors not previously known to be regu-

TABLE I—continued

Description	Accession #	Fold Increase	Description	Accession #	Fold Increase		
Biosynthesis/Enzymes			Cell fate				
cyclooxygenase-2	hCox-2	U04636	175.93	CAGR1	U38810	2.64	
zinc finger protein 267	HZF2	X78925	3.91	Bcl-2 like 11	BCL2L11	AF032457	2.52
alpha-2,8-polysialyltransferase	PST	L41680	3.70	Chromosome associated protein E	hCAP-E	AF092563	2.50
prececuloplasmin (EC 1.16.3.1)	CP	M13699	3.69	pleckstrin homology-like domain A1	PHLDA1	Z50194	2.39
sulfotransferase family 1C, member 1	SULT1C1	AB008164	3.20	Cullin gene family member	Hs-cul-4A	U58090	2.31
Osteoblast specific cysteine rich protein	OSCP	AB008375	2.33	regulatory partner for cdk5 kinase	CDKSP35	X80343	2.27
ADP-ribosyltransferase 3	ART3	U47054	2.29	N-myc downstream regulated	NDR1	D87953	2.11
glycerol-3-phosphate dehydrogenase 2	G3PD2	U36310	2.28	Cell Structure/adhesion			
Retinol dehydrogenase	RODH	U89281	2.23	Chondroitin sulfate Versican	CSPG2	X15998	5.26
Phosphodiesterase 4B	PDE4B	L20971	2.21	M-phase phosphoprotein 11	MPP11	X98260	3.44
Gamma-glutamylcysteine synthetase	GGCS	L35546	2.20	crystallin	CRYA1	X14789	3.35
Hep27	Hep27	U31875	2.19	alpha-catenin	CTNNA2	M94151	3.23
preprocarboxypeptidase A2	proCPA2	U19977	2.18	vitronectin alpha subunit precursor	VNRA	M14648	2.78
Placental Protein 5	PP5	D29992	2.13	Down syndrome cell adhesion molecule	CHD2-42	AF023450	2.67
Cytokines and Chemokines				Bicaudal 1	BICD1	U90030	2.27
GRO2 oncogene	MIP2A	M36820	38.94	cubilin	CUBN	AF034611	2.21
interferon-beta-2	IFN-B2	X04430	11.98	Immune response			
insulin-like growth factor II	IGF2	J03242	9.20	immunoglobulin heavy chain V(H)5	IHC-V(H)5	X58401	7.10
TGFb inducible early protein	TEIG	AF050110	8.67	Human V beta Tcell receptor	TCRVB6S1	U03115	6.94
bone morphogenetic protein 2A	BMP2A	M22489	7.63	Semaphorin E	SEME	AB000220	6.94
Thrombopoietin	TPO	L33410	4.97	CD28 ligand	B7-2	U04343	3.86
CYR61 protein	CYR61	Y11307	4.91	NK-associated transcript 3	NKAT3	X93595	3.59
pituitary tumor transforming gene protein 2	PTTG2	AF095288	4.81	VJ region immunoglobulin	IGL	AF043586	3.47
inhibin beta-B-subunit precursor	INHBB	M31682	3.81	CD69 antigen	CD69	Z22576	3.10
interferon	IFN	V00542	3.61	phospholipase A2 activating protein	PLAP	AJ238243	2.94
growth differentiation factor 5	CDMP1	X80915	3.53	HLA-DRB1	HLA-DRB1	M32578	2.81
heparin-binding epidermal gf-like growth factor	HBGFL	M60278	2.92	Src-like adapter protein	SLAP	D89077	2.76
angiotensin II type 2 receptor	AT2	U20860	2.31	pre-T/NK cell associated protein (3C1)	3C1	L17328	2.71
erythropoietin	EPO	X02158	2.22	PMA induced gene 1	APR	D90070	2.59
leptin	OB	D63710	2.21	CD30 ligand	CD30L	L09753	2.59
tachykinin 2 precursor, isoform beta	TAC2	U37529	2.18	cerebellar degeneration-associated protein	CDR1	M16965	2.51
interferon-related developmental regulator 2	IFNRP	U09585	2.16	complement factor H-related protein 4	FHR-4	X98337	2.17
connective tissue activating peptide III	PPBP	M54995	2.16	MAGE-B2	MAGE-B2	U93163	2.08
keratinocyte growth factor	FGF7	M60828	2.15	Protein Tyrosine phosphatase, Non recpt2	PTPN2	M25393	2.07
RANTES	RANTES	M21121	2.11	C-reactive protein	PTX1	X56692	2.03
Ephrin B2	EPHNB2	U81262	2.07	sialoprotein CD43	CD43	X52075	2.01
prostaglandin E2 receptor	PTGER2	U19487	2.04	Neuro			
endothelial differentiation protein (edg-1)	EDG1	M31210	2.03	proneurotensin/proneurotensin N	NTS	U91618	3.79
Orphan Receptors				synaptobrevin 1 (SYB1)	SYB1	M36200	2.83
TR3 orphan receptor	NPI	L13740	97.28	Uncategorized			
mitogen induced nuclear orphan receptor	MINOR	U12767	25.60	monocyte secretory protein	JE	M28225	8.19
neuron derived orphan receptor	NOR-1	D78579	4.07	tear protein/PRPb homolog	BPLP	S83198	5.26
				lethal (3) malignant brain tumor protein	l(3)mbt	U89358	3.67

lated by E_2 ; these genes are therefore implicated in further E_2 transcriptional action. In some situations, linked gene programs could be tentatively identified, based upon the existing literature. As an example, bone morphogenetic protein 2 (Table I, cytokines) stimulates osteoblast precursor-cell differentiation in part via up-regulating the AREB6 transcription factor (29). Estrogen induces osteoblast differentiation (30), and we found that E_2 stimulates both genes here, via a PI3K-induced mechanism. Another transcriptional target for AREB6 is the $Na^+/K^+-ATPase$ gene (31). E_2 is known to stimulate the activity of this enzyme (32), potentially linking these observations to upstream signaling. As shown here, the HZF2 transcription factor is induced by E_2 in PI3K-dependent fashion (Table I). HZF2 has been reported to be up-regulated by nitric oxide (NO) (33), and E_2 strongly and rapidly stimulates NO production in EC in PI3K-dependent fashion (20). NO induction by E_2 prevents the deleterious blood vessel response to ischemia-reperfusion injury (20). Together, these results potentially identify a linked cell biological program in EC. Several members of the EGR family of transcription factors were identified as induced by E_2 in our array. Egr-1 is up-regulated (and important) in the response to acute and chronic vascular injury, where it may serve a protective function (34, 35). E_2 mitigates the acute injury response to carotid angioplasty (36), perhaps in part through inducing Egr-1 in the EC, as shown here.

Signaling molecules were also rapidly induced by E_2 , including both kinases and phosphatases. Steroid and glucocorticoid-

inducible kinase activity is known to be a target for PI3K/Akt signaling (37) and has roles in both steroid-induced memory (38) and sodium transport (39), which are both functions of E_2 (40, 41). We also found that, via PI3K, E_2 up-regulates *Cot* (*Tpl-2*), a transformation-associated factor and serine/threonine kinase. Akt phosphorylation of Cot induces NF- κ B-dependent transduction (42), important for various functions, including cell survival. E_2 potentially inhibits hypoxia-induced EC apoptosis (14).

Genes coding for structural proteins, cytokines, or enzymes were identified as being stimulated by E_2 . Two members of the NGFI-B subfamily of nuclear orphan receptors, *TR3* and *MINOR*, were rapidly and strongly induced by E_2 via PI3K. A cytokine gene, *CYR61*, was previously demonstrated to be up-regulated by E_2 in breast cancer but through an unknown mechanism (43). In EC, we implicate signaling via PI3K. Up-regulation of the *PTTG* gene by estrogen (found here) was previously shown to contribute to the pathogenesis of E_2 -induced pituitary tumor formation and the stimulation of bFGF secretion (44). E_2 and bFGF are recognized angiogenesis factors (14, 45), and therefore a potential linkage in EC of PTTG-induced bFGF could be important for the recognized neovascularization function of E_2 . The *PLAP* (phospholipase A2-activating protein) gene was found to be stimulated in our array (Table I). This protein has been reported to contribute to up-regulate the *Cox-2* gene and stimulate prostaglandin E_2 (PGE_2) production (46). Based on our results, we propose a

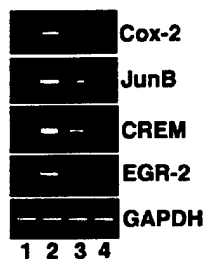


FIG. 3. Validation of the microarray data by RT-PCR. EC were incubated with 10 nM E_2 (lane 2), with 10 nM E_2 and LY294002 (PI3K inhibitor) (lane 3), with LY294002 alone (lane 4), or no treatment (lane 1) for 40 min. Total RNA from the four conditions was then extracted and reverse-transcribed to cDNA. PCR was performed using gene-specific primers (see "Materials and Methods") for *Cox-2*, *JunB*, *CREM*, and *EGR-2*, with glyceraldehyde-3-phosphate dehydrogenase as a control.

linkage of E_2 -induced, PI3K-dependent up-regulation of *PLAP*, contributing to E_2 -induced *Cox-2* up-regulation and PG secretion/production (see below). Thus, many relevant genes are rapidly induced by this sex steroid in response to one signal pathway typically initiated at the plasma membrane.

Confirmation by RT-PCR of E_2 -induced Gene Up-regulation—To confirm the array studies, we carried out semi-quantitative RT-PCR for several of the genes identified (Fig. 3). For the four genes examined (*Cox-2*, *JunB*, *CREM*, and *EGR2*), there was increased expression in EC after 40-min exposure to 10 nM E_2 (lane 2), compared with the control (no E_2) (lane 1). Furthermore, this increase was significantly abrogated by addition of the PI3K inhibitor, LY294002 (lane 3), whereas the inhibitor alone had no effect compared with control levels (lane 4). The results provide validation of the array data for these specific genes.

***Cox-2* Gene and E_2 /ER Interactions Result from Rapid Signaling by the Steroid**—One of the genes strongly up-regulated by E_2 in our EC gene array codes for the *Cox-2* enzyme (Table I). *Cox-2* activity gives rise to PGI_2 and PGE_2 production, important for various aspects of vascular function (47, 48). To explore the interactions between E_2 signaling through PI3K to *Cox-2* in greater depth, we first confirmed the array results by RT-PCR (Fig. 3). We then further investigated transcriptional regulation by E_2 . We therefore expressed in BAEC a plasmid containing a 1.8-kb human *Cox-2* promoter driving a luciferase reporter. This was significantly responsive to 10 nM E_2 , in a PI3K-dependent fashion (Fig. 4).

One known target for PI3K/Akt signaling is the activation of the NF κ B transcription factor (49, 50). We found that a soluble NF κ B inhibitor (but not its inactive control) completely reversed the E_2 stimulation of the *Cox-2* promoter (Fig. 4). These data support the microarray studies and the idea that the up-regulation of the *Cox-2* gene by E_2 is transcriptional. Furthermore, this E_2 action depends on signaling via PI3K and NF κ B. NF κ B binding sites are present within the human *Cox-2* promoter at -580 and -358. Supporting this mechanism, we found that E_2 significantly activated both 0.8 and 0.4 kb *Cox-2* promoters driving luciferase reporter constructs, suggesting that the NF κ B binding site at -358 in particular is important.³ Previous studies indicate that PI3K/Akt can regulate *Cox-2* mRNA production or stability in positive or negative fashion, dependent upon the stimulus and cellular context (51, 52).

***Cox-2* Protein Synthesis and Prostaglandin Secretion Are Stimulated by E_2** —*Cox-2* protein synthesis was then determined by Western blot. After 1-h exposure of EC to E_2 , the

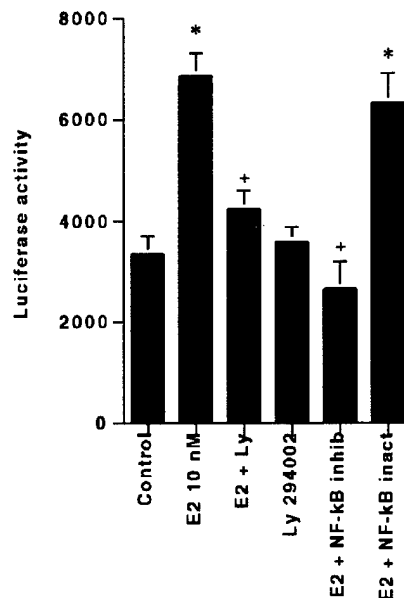


FIG. 4. E_2 stimulates the activity of a *Cox-2* promoter/luciferase reporter via PI3K in endothelial cells. EC were transfected with a plasmid to express a 1.8-kb *Cox-2* promoter plasmid driving the luciferase reporter. The cells were recovered and synchronized and then incubated for 6 h with 10 nM E_2 with or without 10 μ M LY294002, or with 20 μ M of either an NF κ B active inhibitor (SN50M) or an inactive analog (SN50). The bar graph reflects the mean $\pm$ S.E. from triplicate determinations in each of two experiments, combined for analysis. *, $p < 0.05$ for control versus E_2 ; +, $p < 0.05$ for E_2 versus E_2 + LY294002 by analysis of variance plus Scheffe's test.

Cox-2 protein was nearly 3-fold increased in relevant E_2 concentration-responsive fashion (Fig. 5A). The PI3K inhibitors, LY 294002 and wortmannin, each caused an 80% reduction in *Cox-2* protein synthesis, as did ICI 182,780, an ER antagonist. Expression of dominant negative constructs for the p85 subunit of PI3K (DN-PI3K) or Akt (DN-Akt) also resulted in substantial inhibition of E_2 -induced *Cox-2* protein expression. The dominant negative constructs had no effects alone (data not shown).

In preliminary studies, we determined a time course for PGE_2 and PGI_2 secretion in response to E_2 . Compared with basal secretion (0 time), E_2 stimulated an initial 2-fold PGE_2 release at 10 min (first point assessed), reaching a maximum increase at 30 min and plateauing thereafter (data not shown). Based upon these results, BAEC were then incubated with 10 nM E_2 with or without inhibitors of ER, PI3K, and *Cox-1* or *Cox-2* for 30 min. As seen in Fig. 5B, E_2 stimulated a 13-fold increase of PGE_2 secretion, 75% prevented by ICI 182,780. Co-incubation of the cells with NS-398 (a specific *Cox-2* inhibitor) reversed the E_2 effect by 86%. A specific *Cox-1* inhibitor (SC-560) also provided a 33% inhibition of the E_2 effect, suggesting that PGE_2 synthesis was mainly dependent upon the *Cox-2* enzyme. It is recognized that PGE_2 and PGI_2 can both result from either *Cox-1* or *Cox-2* enzymatic action, but in a given cell type or in response to a specific stimulus, one cyclooxygenase activity may predominate over the other (53). Importantly, the PI3K inhibitors, wortmannin and LY 294002 each prevented E_2 -induced PGE_2 secretion by 87%. This is consistent with the ability of E_2 to activate the *Cox-2* gene in a PI3K-dependent fashion, as identified by microarray and reporter studies. Interestingly, the PGE_2 receptor was also found in our array to be up-regulated by E_2 via PI3K (Table I).

As for PGI_2 , E_2 induced a 4-fold increase in secretion (Fig. 5B). This was 60% reversed by ICI 182,780, 58% by the *Cox-2* inhibitor, and 36% by the *Cox-1* inhibitor. The PI3K inhibitors also reduced the E_2 induction of PGI_2 by 60%. Our results

³ A. Pedram, M. Razandi, M. Aitkenhead, C. C. W. Hughes, and E. R. Levin, unpublished observations.

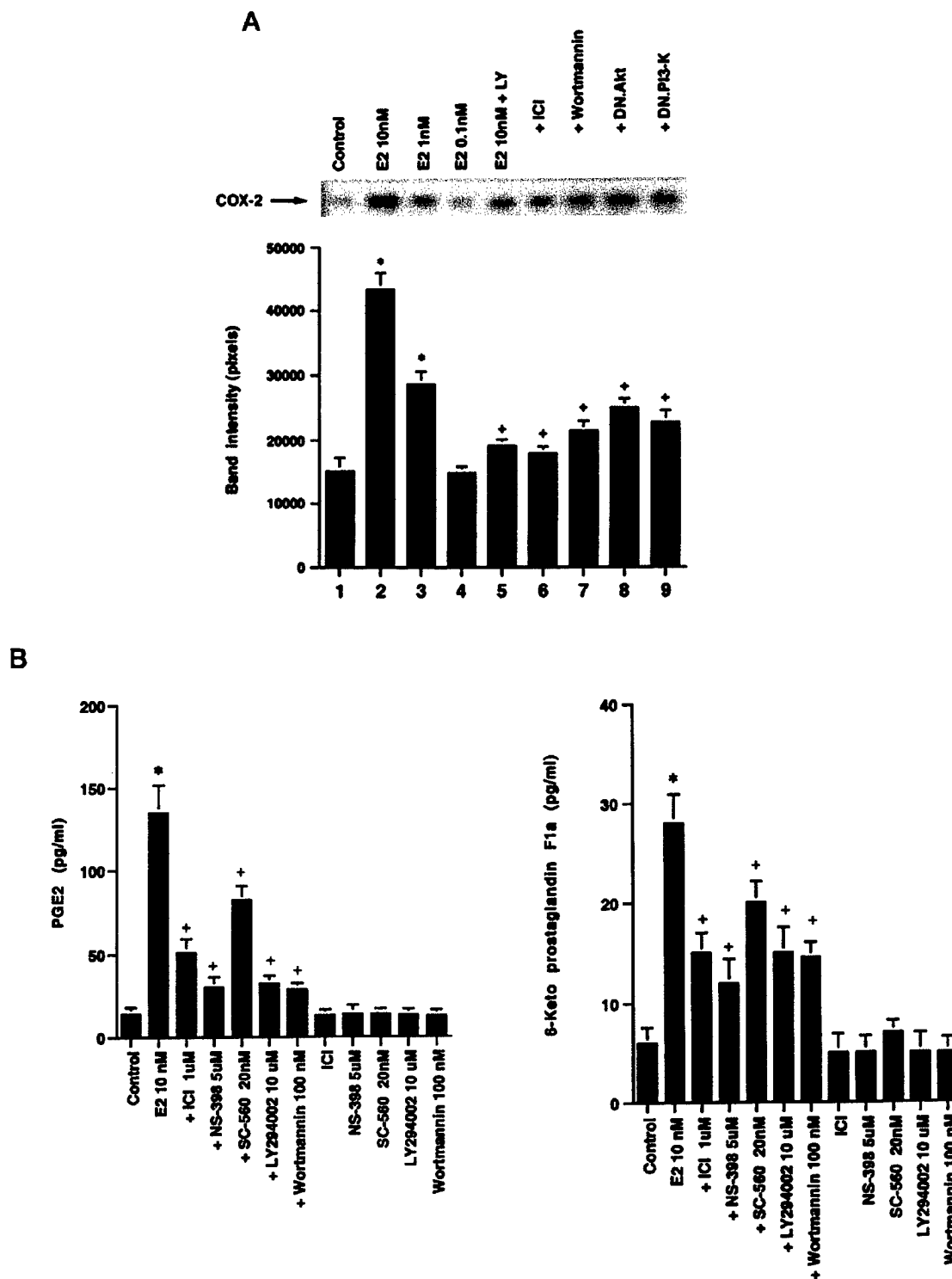


FIG. 5. A, E₂ stimulates the production of Cox-2 protein in EC via PI3K and Akt signaling. EC were incubated with several concentrations of E₂ for 60 min, and Cox-2 protein was determined by Western blot of immunoprecipitated cell lysate. In some conditions, LY294002 or ICI182780 was added 30 min prior to 10 nM E₂, or the cells were first transfected to express pMT2-AH-Akt (*DN.Akt*) or pcDNA3-delta p85 (*DN.PI3-K*). Control and E₂ with or without LY conditions were carried out in cells transfected with pcDNA3. A representative study is shown, and the bar graph reflects three experiments combined. *, $p < 0.05$ for control versus E₂; +, $p < 0.05$ for 10 nM E₂ versus E₂ plus inhibitor. B, secretion of PGE₂ (left) and PGI₂ (6-keto prostaglandin F1alpha) (right) in response to E₂. EC were incubated with 10 nM E₂ with or without PI3K inhibitors, or with E₂ + NS-398 (Cox-2 inhibitor) or SC-560 (Cox-1 inhibitor) for 30 min (soluble inhibitors were added 30 min prior to E₂). The bar graph represents three combined experiments.

greatly extend the observations of others that E₂ can rapidly stimulate the secretion of PGI₂ from endothelial cells (54, 55). Here we show transcriptional up-regulation of the *Cox-2* gene, increased protein production, and stimulation of both PGE₂

and PGI₂ secretion, in a PI3K-dependent fashion. An observation that is relevant for EC is that Cox-2 and cAMP-signaling enhances angiogenesis through the induction of vascular endothelial growth factor (56). Cox-2 and cAMP are up-regulated by

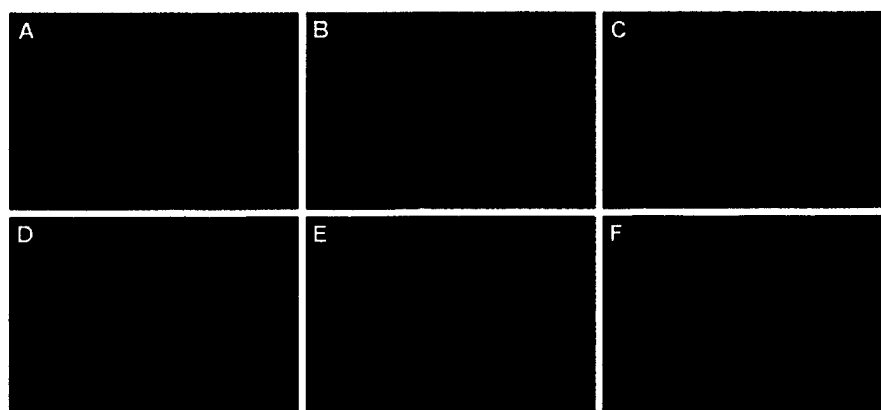
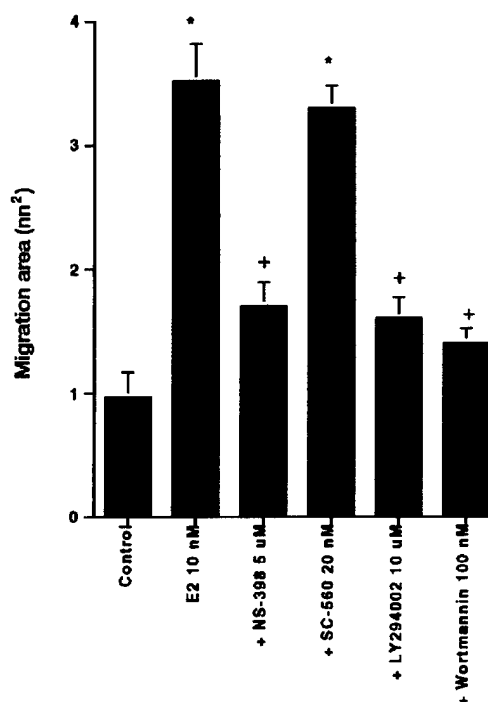


FIG. 6. E_2 stimulates EC migration in PI3K and Cox-2 related fashion. Cultured EC were cut with a surgical blade, and all cells were removed on the left side of the wound with a scraper. The remaining cells were then cultured overnight with/without 10 nM E_2 with or without PI3K and Cox-2 inhibitors. *Panel A* is control cells (no E_2 or serum); *panel B* is E_2 . *Panel C* is E_2 + NS-398, *panel D* is E_2 + SC-560, *panel E* is E_2 + LY294002, and *panel F* is E_2 + wortmannin (50 nM). The inhibitors alone had no effects (data not shown). The bar graph below the composite reflects three experiments combined. *, $p < 0.05$ for control versus E_2 ; +, $p < 0.05$ for E_2 versus E_2 plus inhibitor.



E_2 signaling from the membrane (2, 6, and here). This may be significant to E_2 -modulated induction and developmental function of vascular endothelial growth factor, in the formation and permeability of the blood vessels of the ovary and uterus (56, 57).

EC Migration—We then examined possible roles for Cox-2-derived prostaglandins and E_2 in mediating EC migration. E_2 is known to induce several aspects of angiogenesis, including EC migration (14), and Cox-2 also importantly participates in these processes (28). Cultured EC were “wounded,” and the migration of EC across this wound barrier was determined after 24-h exposure to various conditions. As seen in Fig. 6, 10 nM E_2 (*panel B*) induced a 3-fold increase in migration area compared with control EC (*panel A*). The effect of E_2 was reduced 75% by a Cox-2 inhibitor (*panel C*), but not significantly by a Cox-1 inhibitor (*panel D*). The effect of E_2 was also prevented by LY294002 (*panel E*) and by wortmannin (PI3K inhibitor) (*panel F*). The inhibitors by themselves had no effects on EC migration (data not shown). Thus, we link the Cox-2 up-regulation in EC, induced by E_2 and PI3K signaling, to a cell biological outcome.

Increasingly, the integration of membrane and nuclear actions of steroids is recognized. An important mechanism demonstrated here is that steroids rapidly induce through kinase signaling many genes coding for transcription factors. We determined that the identified transcription factor gene promoters usually lack any form of estrogen response elements. Transcription factor up-regulation presumably then leads to the induction of additional genes that regulate steroid-induced cellular function. However, we also show that, by the same signaling mechanism, steroids rapidly up-regulate genes coding for enzymes or signaling molecules. The protein products of these genes both directly impact cell functions (*i.e.* cell migration) and induce additional transcription. Through membrane-initiated steroid signaling (MISS), we propose that estrogen affects overall cellular processes by post-translationally modulating the functions of existing proteins (9, 14) and by activating discrete programs of gene expression.

REFERENCES

1. Truss, M., and Beato, M. (1993) *Endocrine Rev.* **14**, 459–479
2. Aronica, S. M., Kraus, W. L., and Katzenellenbogen, B. S. (1994) *Proc. Natl. Acad. Sci.* **91**, 8517–8521

3. Tesarik, J., and Mendoza, C. (1995) *J. Clin. Endocrinol. Metab.* **80**, 1438–1443
4. Le Mellay, V., Grosse, B., and Lieberherr, M. (1997) *J. Biol. Chem.* **272**, 11902–11907
5. Pietras, R., and Szego, C. M. (1977) *Nature* **265**, 69–72
6. Razandi, M., Pedram, A., Greene, G. L., and Levin, E. R. (1999) *Mol. Endocrinol.* **13**, 307–319
7. Wyckoff, M. H., Chambliss, K. L., Mineo, C., Yuhanna, I. S., Mendelsohn, M. E., Mumby, S. M., and Shaul, P. W. (2001) *J. Biol. Chem.* **276**, 27071–27076
8. Singer, C. A., Figueroa-Masot, X. A., Batchelor, R. H., and Dorsa, D. M. (1999) *J. Neurosci.* **19**, 2455–2463
9. Razandi, M., Pedram, A., and Levin, E. R. (2000) *Mol. Endocrinol.* **14**, 1434–1447
10. Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E., and Auricchio, F. (1996) *EMBO J.* **15**, 1292–1300
11. Chen, Z., Yuhanna, I. S., Galcheva-Gargova, Z., Karas, R. H., Mendelsohn, M. E., and Shaul, P. W. (1999) *J. Clin. Invest.* **103**, 401–406
12. Kumar, R., Wang, E.-A., Mazumdar, A., Talukder, A. H., Mandal, M., Yang, Z., Bagheri-Yarmand, R., Sahin, A., Hortobagyi, G., Adam, L., Barnes, C. J., and Vadlamudi, R. K. (2002) *Nature* **418**, 654–657
13. Levin, E. R. (2001) *J. Appl. Physiol.* **91**, 1860–1867
14. Razandi, M., Pedram, A., and Levin, E. R. (2000) *J. Biol. Chem.* **275**, 38540–38546
15. Watters, J. J., Campbell, J. S., Cunningham, M. J., Krebs, E. G., and Dorsa, D. M. (1997) *Endocrinology* **138**, 4030–4033
16. Duan, R., Xie, W., Li, X., McDougal, A., and Safe, S. (2002) *Biochem. Biophys. Res. Commun.* **294**, 384–394
17. Duan, R., Xie, W., Burghardt, R. C., and Safe, S. (2001) *J. Biol. Chem.* **276**, 11590–11598
18. Porter, W., Saville, B., Hoivik, D., and Safe, S. (1997) *Mol. Endocrinol.* **11**, 1569–1580
19. Watters, J. J., Chun, T. Y., Kim, Y. N., Bertics, P. J., and Gorski, J. (2000) *Mol. Endocrinol.* **14**, 1872–1881
20. Simoncini, T., Hafezi-Moghadam, A., Brazil, D. P., Ley, K., Chin, W. W., and Liao, J. K. (2000) *Nature* **407**, 538–541
21. Long, A. D., Mangalam, H. J., Chan, B. Y. P., Toller, L., Hatfield, G. W., and Baldi, P. (2001) *J. Biol. Chem.* **276**, 19937–19944
22. Sheng, H., Shao, J., Dixon, D. A., Williams, C. S., Prescott, S. M., DuBois, R. N., and Beauchamp, R. D. (2000) *J. Biol. Chem.* **275**, 6628–6635
23. Khwaja, A., Rodriguez-Vician, P., Wennstrom, S., Warne, P. H., and Downward, J. (1997) *EMBO J.* **16**, 2783–2793
24. Kong, M., Mounier, C., Wu, J., and Posner, B. I. (2000) *J. Biol. Chem.* **275**, 36035–36042
25. Pedram, A., Razandi, M., and Levin, E. R. (September 3, 2002) *J. Biol. Chem.* **277**, 101074fjbc.M202391200
26. Hyder, S. M., Nawaz, Z., Chiapetta, C., Yokoyama, K., and Stancel, G. M. (1995) *J. Biol. Chem.* **270**, 8506–8513
27. Lazennec, G., Alcorn, J. L., and Katzenellenbogen, B. S. (1999) *Mol. Endocrinol.* **13**, 969–980
28. Matsumoto, H., Ma, W.-g., Daikoku, T., Zhao, X., Paria, B. C., Das, S. K., Trzaskos, J. M., and Dey, S. (2002) *J. Biol. Chem.* **277**, 29260–29267
29. Locklin, R. M., Riggs, B. L., Hicok, K. C., Horton, H. F., Byrne, M. C., and Khosla, S. (2001) *J. Bone Miner. Res.* **16**, 2192–2204
30. Plant, A., Samuels, A., Perry, M. J., Colley, S., Gibson, R., and Tobias, J. H. (2002) *J. Cell Biochem.* **84**, 285–294
31. Watanabe, Y., Kawakami, K., Hirayama, Y., and Nagano, K. (1993) *J. Biochem.* **114**, 849–855
32. Dzurba, A., Ziegelhoffer, A., Vrbjar, N., Styk, J., and Slezak, J. (1997) *Mol. Cell Biochem.* **176**, 113–118
33. Schafer, U., Schneider, A., and Neugebauer, E. (2000) *Biochim. Biophys. Acta* **1494**, 269–276
34. Khachigan, L. M., Lindner, V., Williams, A. J., and Collins, T. (1996) *Science* **271**, 1427–1431
35. Du, B., Kent, K. C., Bush, H., Jr., Schulick, A. H., Kreiger, K., Collins, T., and McCaffrey, T. A. (2000) *J. Biol. Chem.* **275**, 39039–39047
36. Mendelsohn, M. E., and Karas, R. H. (1999) *New Engl. J. Med.* **340**, 1801–1811
37. Richards, J. S., Sharma, S. C., Falender, A. E., and Lo, Y. H. (2002) *Mol. Endocrinol.* **16**, 580–599
38. Tsai, K. J., Chen, S. K., Ma, Y. L., Hsu, W. L., and Lee, E. H. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 3990–3995
39. Faletti, C. J., Perrotti, N., Taylor, S. I., and Blazer-Yost, B. L. (2002) *Am. J. Physiol.* **282**, C494–C500
40. Sherwin, B. B. (1998) *Proc. Soc. Exp. Biol. Med.* **217**, 17–22
41. Ediger, T. R., Kraus, W. L., Weinman, E. J., and Katzenellenbogen, B. S. (1999) *Endocrinology* **140**, 2976–2982
42. Kane, L. P., Mollenauer, M. N., Xu, Z., Turck, C. W., and Weiss, A. (2002) *Mol. Cell Biol.* **22**, 5662–5674
43. Tsai, M. S., Bogart, D. F., Li, P., Mehmi, I., and Lupu, R. (2002) *Oncogene* **21**, 964–973
44. Heany, A. P., Fernando, M., and Melmed, S. (2002) *J. Clin. Invest.* **109**, 277–283
45. Lederman, R. J., Mendelsohn, F. O., Anderson, R. D., Saucedo, J. F., Tenaglia, A. N., Hermiller, J. B., Hillegass, W. B., Rocha-Singh, K., Moon, T. E., Whitehouse, M. J., Annex, B. H., and TRAFFIC investigators. (2002) *Lancet* **359**, 2048–2050
46. Ribardo, D. A., Kuhl, K. R., Peterson, J. W., and Chopra, A. K. (2002) *Toxicol.* **40**, 519–526
47. Li, D. Y., Hardy, P., Abran, D., Martinez-Bermudez, A. K., Guerguerian, A. M., Bhattacharaya, M., Almazan, G., Peri, K. G., Varma, D. R., and Chemtob, S. (1997) *Am. J. Physiol.* **273**, R1283–R1290
48. Hoehner, K., Endemann, D., Kammerl, M. C., Grobecker, H. F., and Kurtz, A. (2002) *Br. J. Pharmacol.* **136**, 1117–1126
49. Pandey, S. K., He, H. J., Chesley, A., Juhaszova, M., Crow, M. T., and Bernier, M. (2002) *Endocrinology* **143**, 375–385
50. Koul, D., Yao, Y., Abbruzzese, J. L., Yung, W. K., and Reddy, S. A. (2001) *J. Biol. Chem.* **276**, 11402–11408
51. Monick, M. M., Robeff, P. K., Butler, N. S., Flaherty, D. M., Carter, A. B., Peterson, M. W., and Hunninghake, G. W. (2002) *J. Biol. Chem.* **277**, 32992–33000
52. Tang, Q., Chen, W., Gonzales, M. S., Finch, J., Inoue, H., and Bowden, G. T. (2001) *Oncogene* **20**, 5164–5172
53. Coceani, F., Ackerley, C., Seidltz, E., and Kelsey, L. (2001) *Br. J. Pharmacol.* **132**, 241–251
54. Akaraseenont, P., Techatrasak, K., Thaworn, A., and Chotewuttakorn, S. (2000) *Inflamm. Res.* **49**, 460–465
55. Shareman, T. S., Chambliss, K. L., Gibson, L. L., Pace, M. C., Mendelsohn, M. E., Pfister, S. L., and Shaul, P. W. (2002) *Am. J. Respir. Cell Mol. Biol.* **26**, 610–616
56. Amano, H., Hayashi, I., Yoshida, S., Yoshimura, H., and Majima, M. (2002) *Hum. Cell* **15**, 13–24
57. Ferrara, N., Chen, H., Davis-Smyth, T., Gerber, H. P., Nguyen, T. N., Peers, D., Chislow, V., Hillan, K. J., and Schwall, R. H. (1998) *Nat. Med.* **4**, 336–340

Proximal Events in Signaling by Plasma Membrane Estrogen Receptors*

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Mahnaz Razandi‡, Ali Pedram‡, Steven T. Park‡, and Ellis R. Levin‡§¶

From the Division of Endocrinology, Veterans Affairs Medical Center, Long Beach, Long Beach, California 90822
and the Departments of ‡Medicine and §Pharmacology, University of California, Irvine, California 92717

Estradiol (E2) rapidly stimulates signal transduction from plasma membrane estrogen receptors (ER) that are G protein-coupled. This is reported to occur through the transactivation of the epidermal growth factor receptor (EGFR) or insulin-like growth factor-1 receptor, similar to other G protein-coupled receptors. Here, we define the signaling events that result in EGFR and ERK activation. E2-stimulated ERK required ER in breast cancer and endothelial cells and was substantially prevented by expression of a dominant negative EGFR or by tyrphostin AG1478, a specific inhibitor for EGFR tyrosine kinase activity. Transactivation/phosphorylation of EGFR by E2 was dependent on the rapid liberation of heparin-binding EGF (HB-EGF) from cultured MCF-7 cells and was blocked by antibodies to this ligand for EGFR. Expression of dominant negative mini-genes for $G\alpha_q$ and $G\alpha_i$ blocked E2-induced, EGFR-dependent ERK activation, and $G\beta\gamma$ also contributed. G protein activation led to activation of matrix metalloproteinases (MMP)-2 and -9. This resulted from Src-induced MMP activation, implicated using PP2 (Src family kinase inhibitor) or the expression of a dominant negative Src protein. Antisense oligonucleotides to MMP-2 and MMP-9 or ICI 182780 (ER antagonist) each prevented E2-induced HB-EGF liberation and ERK activation. E2 also induced AKT up-regulation in MCF-7 cells and p38 β MAP kinase activity in endothelial cells, blocked by an MMP inhibitor, GM6001, and tyrphostin AG1478. Targeting of only the E domain of ER α to the plasma membrane resulted in MMP activation and EGFR transactivation. Thus, specific G proteins mediate the ability of E2 to activate MMP-2 and MMP-9 via Src. This leads to HB-EGF transactivation of EGFR and signaling to multiple kinase cascades in several target cells for E2. The E domain is sufficient to enact these events, defining additional details of the important cross-talk between membrane ER and EGFR in breast cancer.

Steroid hormones such as estrogen are essential to the development and reproductive functions of prokaryotic and eukaryotic organisms. Traditionally, steroid hormone action was

exclusively attributed to the binding of nuclear receptors and the subsequent transactivation of target genes that led to cell biological effects (1). More recently, it has become clear that steroids rapidly act on cells, in seconds to minutes, effects that are classified as “nongenomic” (reviewed in Ref. 2). For estrogen, this has been attributed in most cells to binding a population of receptors that exists within caveolar rafts and other domains in the plasma membrane (3–5). It is at the plasma membrane that estradiol (E2)¹-liganded estrogen receptors (ER) physically associate with the scaffold protein, caveolin-1 (5), and a variety of proximal signaling molecules, including G proteins (6, 7), Src and Ras (8, 9), and B-Raf (10). This results in the activation of cascades of signal transduction, mainly evolving from G protein activation. Comparable with many other G protein coupled receptors (GPCR), G protein activation by ER (6, 7) leads to the stimulation of phospholipase C (11), protein kinase C (12), ERK (9), and phosphatidylinositol 3-kinase and nitric-oxide synthase (13). These positive signaling effects are cell context-specific, and in some cells, estrogen inhibits cytokine-related signal transduction to cell differentiation, proliferation, migration, or cell death (14–17).

What is the nature of the membrane ER, and how does it enact signal transduction? Current evidence favors the idea that the membrane and nuclear ER are the same protein. Antibodies directed against many epitopes of the classical ER α receptor identify membrane ER by immunocytochemistry (18). Expression of antisense DNA to the “nuclear” ER also abrogates the detectable expression of membrane ER in cells containing endogenous receptor (19). In CHO cells, expression of a single cDNA for either ER α or ER β produces both membrane and nuclear receptor populations and results in E2 activation of signal transduction from the membrane (6). In many cell types, endogenous membrane ER have been identified (15, 18, 20) and appear to reflect the localization of receptors that also have the capacity to translocate to the nucleus. The structural aspects of the membrane ER that allow it to activate signaling molecules are not well defined. Assuming that the sequence of the nuclear receptor is the same as the membrane ER, there is no catalytic or kinase sequence inherent to the structure. Recent evidence favors the idea that the E domain of the membrane ER is essential (and perhaps sufficient) for activation of the ERK cascade (5), leading to cell survival (17). Additionally, the AF-1 domain of ER α has been identified to interact with the adapter protein, Shc, in whole cell homogenates (21). Thus, the

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¶ To whom correspondence should be addressed: Medical Service (111-I), Long Beach VA Medical Center/UC-Irvine, 5901 E. 7th St., Long Beach, CA 90822. Tel.: 562-826-5748; Fax: 562-826-5515; E-mail: ellis.levin@med.va.gov.

¹ The abbreviations used are: E2, estradiol; ER, estrogen receptor(s); EGF, epidermal growth factor; EGFR, EGF receptor(s); GPCR, G protein-coupled receptor(s); MMP, matrix metalloproteinase(s); PLC, phospholipase C; PKC, protein kinase C; HB-EGF, heparin-binding epidermal growth factor; TGF, transforming growth factor; EC, endothelial cell(s); CHO, Chinese hamster ovary; ASO, antisense oligonucleotide(s); MSO, scrambled antisense oligonucleotide(s).

membrane ER acts similarly to many other GPCR that also lack catalytic or kinase domains yet signal to important events in cell biology.

As a GPCR, the membrane ER associates with and activates several G proteins. In transfected CHO cells, membrane ER α or ER β co-precipitates with and activates G α_s and G α_q proteins (6). This leads to the expected downstream signaling to cAMP and inositol 1,4,5-trisphosphate generation, signaling that has been shown in cells expressing endogenous ER (22, 23). In EC, endogenous membrane ER physically associates with G α_i and activate endothelial nitric-oxide synthase; this probably takes place within caveolae (7). Additionally, it has been proposed in breast cancer cells that E2/ER transactivates the epidermal growth factor receptor (EGFR), leading to the downstream signaling to ERK activation (24, 25). This occurs through the activation of G $\beta\gamma$, the liberation of heparin-binding EGF (HB-EGF), which results in the binding and activation of the EGFR, and the subsequent stimulation of the ERK signaling cascade. In some of these respects, the membrane ER acts similarly to a wide range of GPCR (26). However, it was further proposed in breast cancer cells that E2 in some undefined way activates the orphan GPCR, GPR30, to stimulate signaling, and this interaction does not require ER (25). These latter data are not in concert with many studies from other laboratories, indicating that E2 requires an ER for signaling from the membrane in various cell types (5, 6, 8, 20, 27, 28).

The utilization of EGFR by E2/ER to signal results from a linked series of events involving multiple upstream molecules, only some of which have been defined. For instance, we do not know the range of G proteins that can be activated to cross-talk to EGFR activation, and it is not clear what signals immediately downstream of G proteins are important. Src participates in the transactivation of EGFR in response to other GPCR ligands and is probably upstream of HB-EGF shedding (29), but its exact role and requirement for ER signaling is unclear. Furthermore, although matrix metalloproteinase (MMP) activation is required for HB-EGF liberation (and subsequent EGFR activation), the identity of the required MMP(s) is mainly undefined, especially as regards ER signaling. These issues are addressed in the studies described here. Finally, much of the interaction between GPCRs and EGFR has examined ERK activation. Thus, we sought additional signaling molecules in several cell types and the structural requirements within ER that utilize this interactive mechanism following endogenous ER ligation by E2.

EXPERIMENTAL PROCEDURES

Materials—Antibodies and substrate for kinase activation/activity were from Santa Cruz Biotechnology (Santa Cruz, CA). PD 98059 was a generous gift from Dr. Alan Saltiel (Parke-Davis). LipofectAMINE was from Invitrogen. Primary cultures of bovine aortic EC were prepared and used as previously described (30). In transfection studies, EC were generally used in passages 4 and 5, based upon the previous observation that this greatly increases the transfection efficiency of these cells. Breast cancer cell lines were obtained from ATCC. The cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 or RPMI 1640 with serum until 48 h prior to experimentation, when they were placed in serum-free conditions and in medium without phenol red. Gelatin was from Sigma, and kinase substrates were from Upstate Biotechnology, Inc. (Lake Placid, NY) or Santa Cruz Biotechnology. PP2, Src family kinase inhibitor, and GM6001, a matrix metalloproteinase (MMP) inhibitor, were from Calbiochem (San Diego, CA).

Kinase Activity Assays—For ERK or p38 β activity assays, the cells were synchronized for 24 h in serum- and growth factor-free medium. The cells were then exposed to E2 for 8 (ERK) or 15 (p38) minutes, with or without additional substances, as previously described (30, 33). The cells were lysed, and lysate was immunoprecipitated with protein A-Sepharose conjugated to antiserum for p38 or ERK. Immunoprecipitated kinases were washed and then added to the proteins ATF-2 (for p38) or myelin basic protein (for ERK) for *in vitro* kinase assays. This

was followed by SDS-PAGE separation and autoradiography/laser densitometry. In addition, the E2-induced phosphorylation of AKT kinases at 10 min was determined to assess activation. Cultured cell lysates were pelleted and dissolved in SDS sample buffer, boiled, separated, and then transferred to nitrocellulose. Phosphorylated kinase proteins were detected using phospho-specific monoclonal antibodies (Santa Cruz) and the ECL Western blot kit (Amersham Biosciences). Equal samples from the cells were also immunoprecipitated, and immunoblots of the precipitated kinase protein from each experimental condition were determined to show equal gel loading. All of the experiments were repeated two or three times.

Transient Transfections—MCF-7, HCC-1569, ZR-75-1, or bovine aortic endothelial cells (passages 4 and 5) were grown to 40–50% confluence and then transiently transfected with 1.5 μ g (each well of 6-well plates) or 10 μ g of fusion plasmid DNA (100-mm dishes). Plasmids included wild type mouse ER α (31) (kindly provided by Dr. Ken Korach) PRK5-HER, a dominant negative EGF receptor construct (kindly provided by Dr. A. Ullrich (32), a dominant negative Src construct, pRC-csrc-K298M (kindly provided by Drs. Louis Luttrell and Robert Lefkowitz (26), a dominant negative, truncated β -adrenergic receptor kinase plasmid (BARK1-CT PRK5) from Dr. Walter Koch (34), and truncated G α subunit plasmids, serving as specific dominant negative constructs for G α_s , G α_q , G α_i , G α_{12} , and G α_{13} (35). Transfection was carried out using LipofectAMINE (Invitrogen). The cells were incubated with liposome-DNA complexes at 37 °C for 5 h, followed by overnight recovery in culture medium containing 10% fetal bovine serum, 24 h of synchronization in serum-free medium, and then treatment with E2 with or without other substances.

Gelatin Zymography Substrate Cleavage and Antisense Studies for MMP Activity—MMP activity, as secreted into the medium of cultured MCF-7 cells, was analyzed by substrate gel electrophoresis (zymography). The cells were synchronized in serum-free medium for 24 h and then incubated in medium with or without 10 nM estrogen for 2 min at 37 °C in a CO $_2$ incubator. The cell medium was removed, concentrated 20-fold by ultrafiltration, and mixed with native gel sample buffer (Bio-Rad), and the proteins were separated by electrophoresis on an 8% gel co-polymerized with 1 mg/ml gelatin (Sigma). Active MMP-2 and MMP-9 (Calbiochem) was loaded into additional lanes on the gel. After electrophoresis, the gels were washed in 2.5% Triton X-100 at room temperature for 1 h and incubated for 16 h at 37 °C (in 0.05 M Tris, pH 7.5, 5 mM CaCl $_2$, 0.02% NaN $_3$). The gel was stained with 0.5% Coomassie Blue and destained in 10% acetic acid, 10% propanol. The study was repeated twice. Gelatinolytic activity appears as a clear band on a blue background. For the fluorescent substrate assay, MCF-7 cells were synchronized for 24 h and then incubated without or with 10 nM estrogen for 2 min. The incubation medium was concentrated 10-fold, and 1 ml of assay buffer (100 mM Tris, pH 7.5, 100 mM NaCl $_2$) containing 5 μ M of the Mca-Pro-Leu-Dpa-Ala-Arg-NH $_2$ substrate for MMP-2/MMP-9 was added and then incubated at 37 °C for 3 h. Excitation at 328 nm and emission at 393 nm were determined in a fluorimeter. To implicate MMP-2 and MMP-9 in the shedding of HB-EGF, the cells were incubated with antisense (ASO) or scrambled antisense (MSO) with the same base composition for each of the two MMPs. The oligonucleotides were: MMP-2, ASO, CCGGGCCATTAGCGCTCCAT, and MSO, TCACCGCGGTACGCATGCCCT; and MMP-9, ASO, CAGGGGCTGC-CAGAGGCTCAT, and MSO, GCGAGCTAGGACTGTGCAGCC. The oligonucleotides were added with LipofectAMINE for 5 h, and the cells were recovered overnight and synchronized in the absence of serum for 12 h. Transfection efficiency exceeded 60%, based upon co-expression of PEGFPc2. Western blot studies were carried out to confirm the efficacy of the ASO but not the MSO to inhibit specific protein production. Studies of E2-induced signaling were then carried out in cells expressing the various oligonucleotides.

Western Blot for HB-EGF and EGFR Phosphorylation—Subconfluent, transfected, or nontransfected cultured bovine aortic endothelial cells were serum-deprived for 24 h and then incubated under various conditions for 10 min with inhibitors followed by 10 min of treatment with stimulants. This included several 17- β -E2 concentrations, ICI 182780 (1 μ M), and 100 nM GM6001, a broad MMP inhibitor. The cells were lysed, and antibodies to HB-EGF or EGFR (tyrosine 1138) (1:50 dilution) were conjugated to Sepharose beads and then added to the cell lysate for 2 h at 4 °C. After pelleting and washing, the samples were electrophoretically separated on a 7% SDS gel, transferred to nitrocellulose, and immunoblotted. Detection utilized the ECL kit (Amersham Biosciences).

RESULTS

Activation of ERK by E2 Requires an ER and the Activation of EGFR by HB-EGF—We first established that E2 required both the presence of an ER and the activation of EGFR to signal to ERK. HCC-1569 cells lack ER, and the cells did not respond to E2 with ERK activation (Fig. 1A, lanes 1 and 2). When ER α was expressed in these cells, 17- β -E2 (lane 4), but not 17- α -E2 (lane 8), was capable of activating ERK, and this was substantially blocked by the ER antagonist, ICI182780 (lane 5). As a positive control, these cells express the EGFR and appropriately respond to EGF (lane 7). The requirement of ER is similar to our previous findings in CHO-K1 cells (6). We then asked whether E2 activation of ERK depends upon EGFR tyrosine kinase activity. We examined this in MCF-7 and ZR-75-1 breast cancer cells and EC (all with ER). Tyrphostin AG1478, specifically directed against the EGFR tyrosine kinase function, prevented EGFR-induced ERK activation in both MCF-7 and ZR-75-1 cells (Fig. 1B, left and center panels). Importantly, tyrphostin AG1478 also substantially prevented the ability of E2 to activate ERK in the three cell types (Fig. 1B, all panels, lanes 2 versus lanes 6). To corroborate this finding, we expressed a dominant negative EGFR (31) in MCF-7 cells, and E2 was much less effective in stimulating this MAP kinase, compared with cells expressing the empty vector (control) (Fig. 1C).

What ligand for EGFR is involved in the transactivation of this receptor by E2? Although there are many members of the EGF family that can bind the EGFR, HB-EGF has often been implicated in the setting of GPCR signaling via this receptor (36). To examine this, we first determined whether E2 could stimulate the secretion of HB-EGF, determined by Western blot. As seen in Fig. 2A, E2 dose-responsively induced a significant enhancement of HB-EGF shedding/secretion from the MCF-7 cells after 3 min of incubation. This was prevented by ICI182780 and by GM6001, an MMP inhibitor. To determine that HB-EGF was the important ligand for EGFR signaling to ERK, we incubated the MCF-7 cells with 10 nM E2, in the presence or absence of antibody to HB-EGF. In the setting of this added antibody, E2 could not significantly activate ERK (Fig. 2B). In contrast, antibody to TGF α -1, another ligand for the EGFR, had no effect on E2-induced ERK, and the antibodies by themselves did not affect basal ERK activity. Similarly, antibody to HB-EGF (but not to TGF α -1) prevented E2-induced phosphorylation of the EGFR (Fig. 2C). Identical findings were determined from EC incubated with E2 (data not shown). These results support the interactions of secreted HB-EGF with EGFR, leading to ERK activation in breast cancer and vascular cells. The data also support ER-mediated, MMP-dependent release of HB-EGF.

Matrix Metalloproteinases 2 and 9 Are Activated and Are Necessary for Signaling by E2—Current evidence supports the idea that GPCRs activate MMP activity, thereby liberating HB-EGF from the cell matrix, leading to the transactivation of the EGFR (36, 37). Therefore, MMP activation represents the step immediately upstream of HB-EGF liberation. In many cell paradigms, including E2 action, the precise MMP(s) activated by GPCR signaling are unknown. We therefore showed that E2 activates MMP activity by demonstrating that the incubation medium from MCF-7 cells treated with E2 for 2 min induces the cleavage of substrate specific for MMP-2 and MMP-9 (Fig. 3A). In contrast, substrate specific for MMP-13 or MMP-3 was not cleaved by the E2-treated cell medium (data not shown), even though breast cancer cells produce these proteolytic enzymes. We then sought to further identify the MMPs by carrying out gelatin zymography. E2 treatment of the cultured MCF-7 cells for 2 min led to the increased secretion and activation of MMP-2 and -9 (Fig. 3B, first and second lanes). To

corroborate the identify of the digested gelatin band activities, active MMPs (Calbiochem) were also run in parallel on a separate gel (data not shown). Functionally, activation of MMP activity was necessary for E2-induced ERK. This was shown in that the MMP inhibitor completely reversed the ability of E2 to activate ERK in both MCF-7 and ZR-75-1 cells (Fig. 3C, left and right panels). This compound did not affect EGF-induced ERK activation, supporting the idea that MMP-related events occur upstream to EGFR activation in this pathway.

Although E2 activates these two MMPs, it is not clear that they are responsible for E2-induced HB-EGF shedding. We therefore used ASO or MSO, with the latter comprised of the same base composition as the ASO for MMP-2 and MMP-9, and expressed them in MCF-7 cells. First, we validated the constructs by showing that the ASO (but not the MSO) for MMP-2 or MMP-9 inhibited the respective protein production in a dose-related manner (Fig. 4A, left panel). Similarly, we validated the function of the MMP-2 or MMP-9 to specifically inhibit only the intended protein target (Fig. 4A, right panel). Using these ASO and MSO, we next determined whether MMP-2 and MMP-9 each contributed to HB-EGF shedding and ERK activation (Fig. 4, B and C). Each ASO significantly down-regulated E2-induced HB-EGF liberation, and expressing the ASO to both MMPs completely blocked this E2 action. The ASO to MMP-2 almost completely prevented the ability of E2 to activate ERK in MCF-7 cells, whereas the ASO to MMP-9 was also substantially able to prevent this signaling; neither MSO had any effect, and the results were similar to those in EC. E2/ER stimulation of MMP-2 and MMP-9 may therefore underlie several important actions in breast cancer, including signaling through ERK to cell proliferation and survival (5, 9). Metalloproteinase activation also contributes to the disengagement of cells from matrix, a necessary initial step preceding invasion and migration behaviors (38). MMP-2 and MMP-9 are well recognized to contribute to these events in various contexts (38, 39).

Specific G Proteins Are Involved in E2-induced Transactivation of EGFR—It has previously been established that E2 can activate G α_s and G α_q , as well as G α_i in several cell models (6, 7). Therefore, one or more G proteins activated by E2 could ultimately result in EGFR signaling to ERK. To examine this issue, we expressed mini-genes for G α subunits of G α_s , G α_i , G α_{12} , G α_{13} , and G α_q constructs that have been shown to act as dominant negatives for specific endogenous G protein subunit activation (35). As seen in Fig. 5A, ERK activation in response to E2 in cells expressing the control plasmid, G α_{tr} (lane 3), was substantially prevented after expressing the inhibitory mini-genes for G α_i and G α_q (lanes 4 and 5). However, dominant negative constructs for the α subunits of G α_s , G α_{12} , and G α_{13} had insignificant effects on this signaling. We also expressed a C-terminal truncated β -adrenergic receptor kinase, pRK5-BARK1-(495–689), that inhibits G $\beta\gamma$ signaling (33). Expression of this construct significantly but incompletely prevented the ability of E2 to activate ERK and HB-EGF liberation (Fig. 5B). Upon expressing ER α in HCC-1569 cells, E2 could now activate ERK in a G α_i , G α_q , and G $\beta\gamma$ -dependent fashion (Fig. 5C). Therefore, both G α and G $\beta\gamma$ subunits contribute to the ability of E2/ER to activate the signaling pathway that ultimately results in EGFR transactivation.

Calcium, PLC, and PKC Activities Mediate E2-induced MMP Activation—The signaling through the identified G proteins potentially leads to the activation of MMP activity and the subsequent downstream signaling through EGFR. We examined which signal pathways immediately downstream of G protein activation that we identified here could mediate MMP activation. E2-induced MMP activity was significantly inhib-

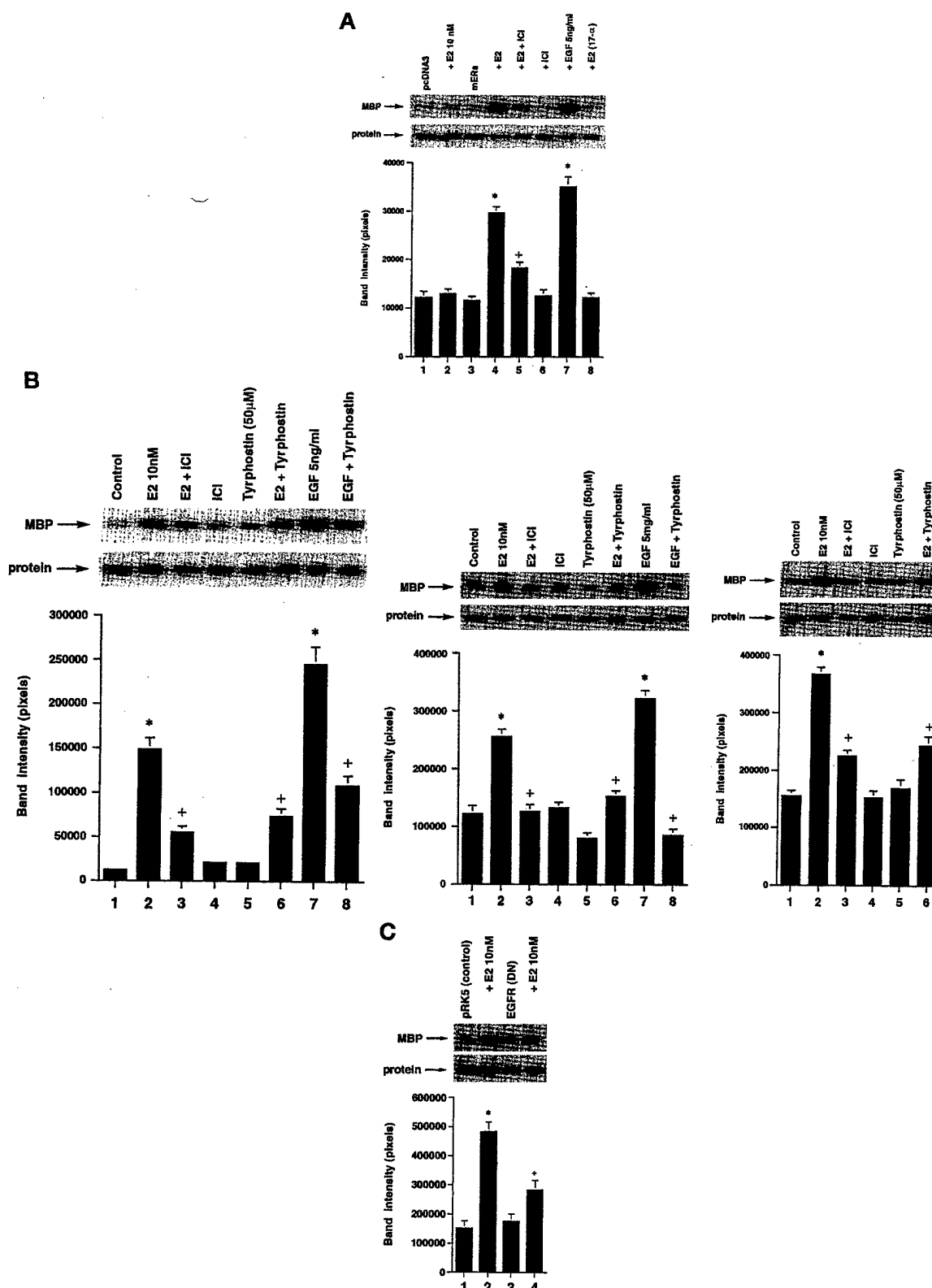


FIG. 1. E2 activates ERK via ER and EGFR. **A**, 17- β -E2 activates ERK only when ER is present. HCC-1569 cells (ER negative) were incubated with 10 nM 17- β -E2 or were transfected to express wild type mouse ER α (mER α) and then incubated with 17- β -E2 or 17- α -E2, and ERK activity (against myelin basic protein) was determined after 8 min in an *in vitro* tube assay as described under "Experimental Procedures." Immunoblots of total ERK protein are shown below the activity. The bar graph represents three combined experiments. *, $p < 0.05$ for control versus E2 or EGF; +, $p < 0.05$ for E2 versus E2 with ICI182780 (ER antagonist). **B**, inhibition of EGFR tyrosine kinase function with tyrphostin AG1478 prevents E2-induced ERK activation in MCF-7 cells (left panel), in ZR-75-1 cells (center panel), or in endothelial cells (right panel). The cells were incubated as described above with 17- β -E2 with or without a specific EGFR tyrosine kinase inhibitor, and ERK activity was determined. Each bar graph represents three combined experiments. *, $p < 0.05$ for control versus E2 or EGF; +, $p < 0.05$ for E2 versus E2 with ICI182780 (ER antagonist) or E2 or EGF versus either E2 or EGF with AG1478 (tyrphostin). **C**, expression of a dominant negative EGFR (EGFR (DN)) prevents E2-induced ERK activation in MCF-7 cells. The cells were transfected to transiently express PRK5-HER dominant negative EGFR, recovered overnight in 10% serum, and 24 h after cell recovery, E2 activation of ERK was determined after 8 min of incubation. *, $p < 0.05$ for control versus E2; +, $p < 0.05$ for E2 versus PRK5-HER transfected cells incubated with E2.

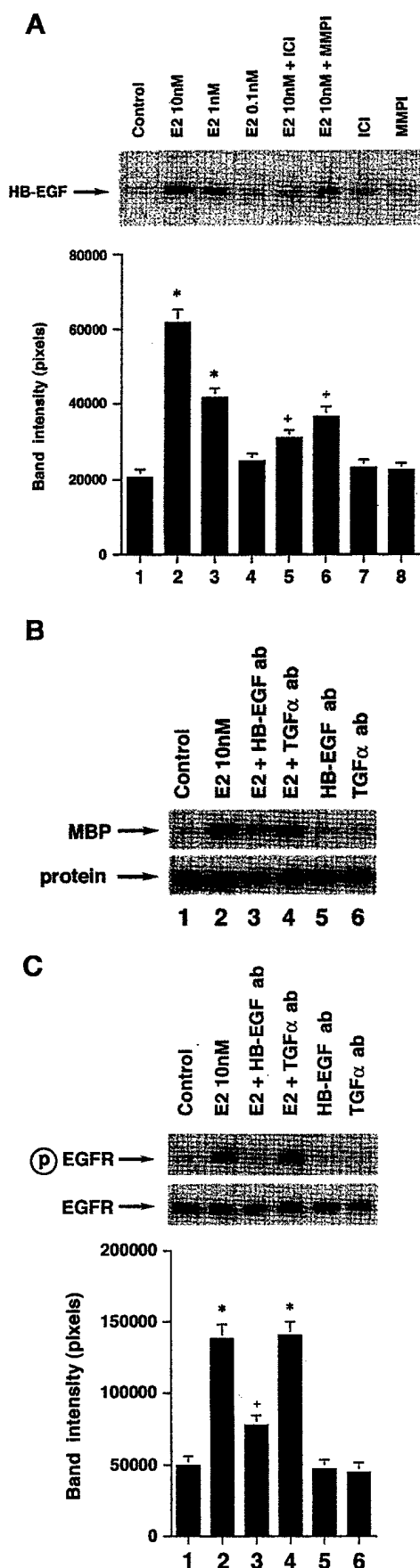


FIG. 2. E2 rapidly stimulates HB-EGF release via matrix metalloproteinase action and ER. **A**, MCF-7 cells were incubated with E2, 0.1–10 nM with or without ICI182780, or 100 nM GM6001, an MMP inhibitor, for 3 min. The HB-EGF shed into the medium was deter-

mined by EDTA, an extracellular calcium chelator, but was not affected by BAPTA-AM (Fig. 2A). This indicates that calcium entry through surface channels, but not the mobilization of intracellular calcium, contributes to E2-induced MMP activation. It has previously been shown that E2 activates several calcium channels that lead to an influx of calcium into the cell (40, 41), and this can result from G_{α_q} or $G_{\beta\gamma}$ activation.

We also found that soluble inhibitors of PLC and PKC (calphostin C and U-73122, respectively) significantly prevented E2 activation of MMP activity (Fig. 3A). This is consistent with our identification here of G_{α_q} and $G_{\beta\gamma}$ as mediating E2-induced ERK activation, because PLC and PKC up-regulation results from the activation of these G protein subunits. We previously showed that E2 can activate G_{α_q} , PLC, and inositol 1,4,5-trisphosphate generation via membrane ER (6), and E2 has been described to stimulate PKC activity in several cell types (reviewed in Ref. 42). PKC-dependent signaling in growth plate chondrocytes mediates E2-induced regulation of these cells, and originates from membrane action of the steroid (43). These findings link the most proximal signaling events to later events (MMP activation and HB-EGF shedding), mediating EGFR transactivation.

Role of Src in Shedding of HB-EGF—It has been documented that E2-liganded ER complex with and activate the Src tyrosine kinase, and this is necessary for E2 stimulation of ERK (8, 17). Src could potentially play a role both upstream and downstream of EGFR activation. We therefore determined where Src activation is required for the proximal signaling induced by E2, leading to EGFR transactivation. As shown in Fig. 3B, E2-induced MMP-2 and MMP-9 activation and secretion at 2 min (first lane versus second lane). This was substantially prevented by the Src family kinase inhibitor, PP2 (third lane), or by expressing a specific dominant negative Src construct, pRC-csrc-K298M (26) (fifth and sixth lanes compared with first and second lanes). Thus, these results define a novel role for Src in E2-induced signaling from the membrane, and we suggest that this molecule may play a similar role in other GPCR-induced activation of EGFR through this mechanism.

ER Is Required for Proximal Signaling Events—We earlier showed that E2 requires an ER to activate ERK (Fig. 1). To further support the idea of the necessity of ER presence for E2 action, we expressed ER α in HCC-1569 cells and determined the proximal signaling events implicated. We first demonstrated that expression of the dominant negative G_{α_i} and G_{α_q} mini-genes substantially blocked E2-induced ERK, compared with kinase activity in the presence of the control (inactive) construct, $G_{\alpha_{ir}}$ (Fig. 6A). Similarly, expression of C-terminal truncated β -adrenergic receptor kinase (BARK1) also down-regulated E2/ER-induced ERK, whereas the truncated mini-gene for $G_{\alpha_{13}}$ was without effect (similar to control). We also examined HB-EGF secretion and found that E2 stimulated the secretion of this receptor ligand only when ER was expressed (Fig. 6B, lanes 1 and 2 versus lanes 3 and 4). ICI182780 and MMP inhibition significantly prevented the stimulation of HB-EGF secretion. Finally, we found that in the presence (but not

mined by Western blot. The bar graph is three experiments combined. *, $p < 0.05$ for control versus E2; +, $p < 0.05$ for E2. **B**, antibody to HB-EGF but not TGF α blocks E2-induced ERK activation. MCF-7 were incubated with 10 nM E2 with or without 10 μ g/ml antibody to HB-EGF or TGF α , and ERK activity was determined after 8 min. The antibodies alone had no effect on ERK activity. **C**, HB-EGF but not TGF α antibody blocks the E2-induced transactivation/phosphorylation of EGFR. The cells were incubated with 10 nM E2 with or without antibodies for 8 min, and lysate was subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an antibody to tyrosine 1173 of the EGF receptor. *, $p < 0.05$ for control versus E2 or E2 plus TGF α antibody; +, $p < 0.05$ for E2 versus E2 with HB-EGF antibody.

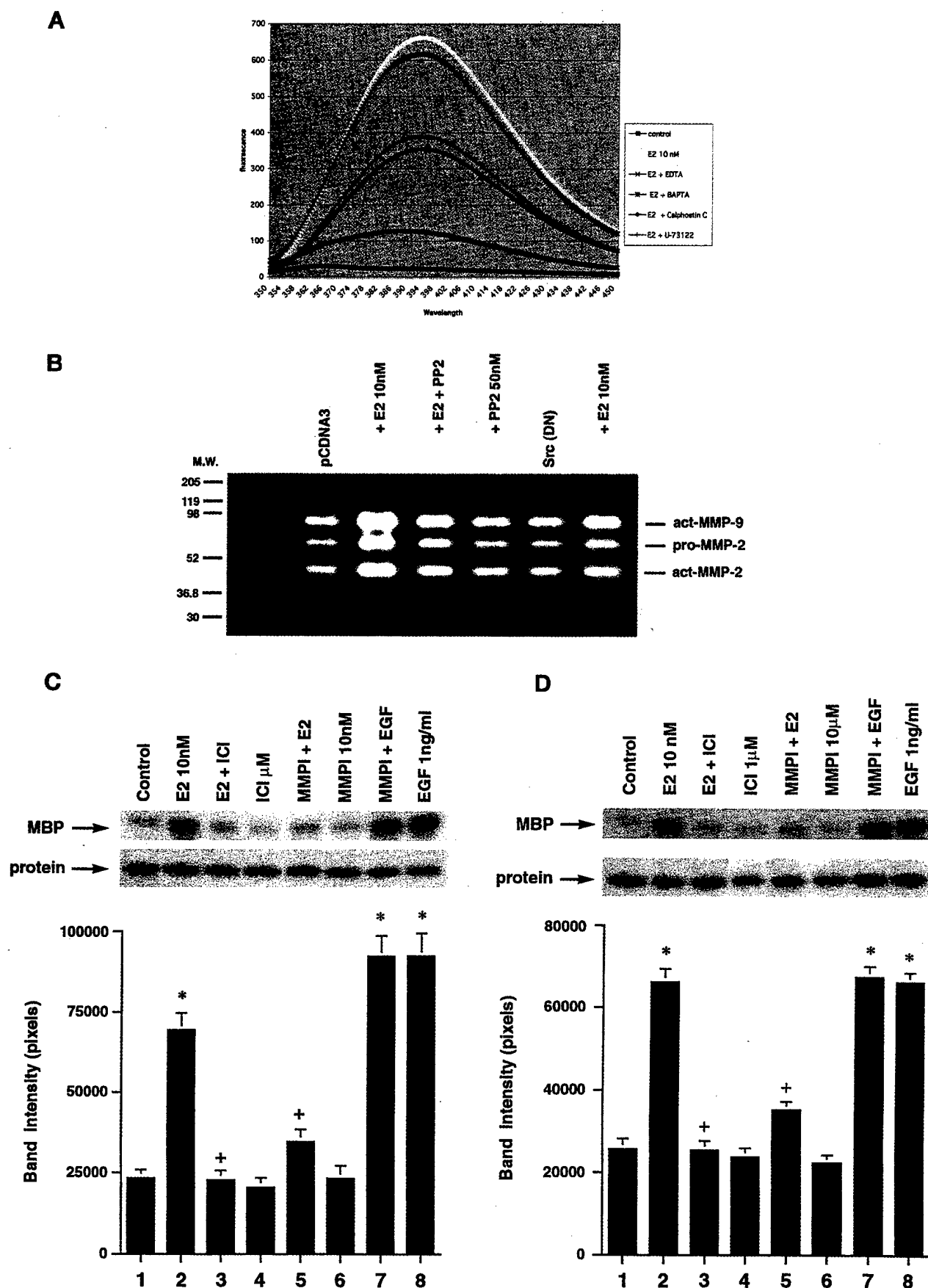


FIG. 3. E2 activates matrix metalloproteinase 2 and 9 secretion and activity. *A*, cells were incubated with or without E2 with or without BAPTA (intracellular calcium inhibitor), EDTA (chelates extracellular calcium), a specific PLC inhibitor, U73122, or a specific PKC inhibitor, calphostin C for 2 min. Cleavage of substrate for MMP-2/MMP-9 by the medium from MCF-7 cells incubated with 10 nM E2 for 2 min was determined by spectrofluorimetry. The data are from triplicate determinations in a representative experiment, repeated twice. *B*, MCF-7 cells were

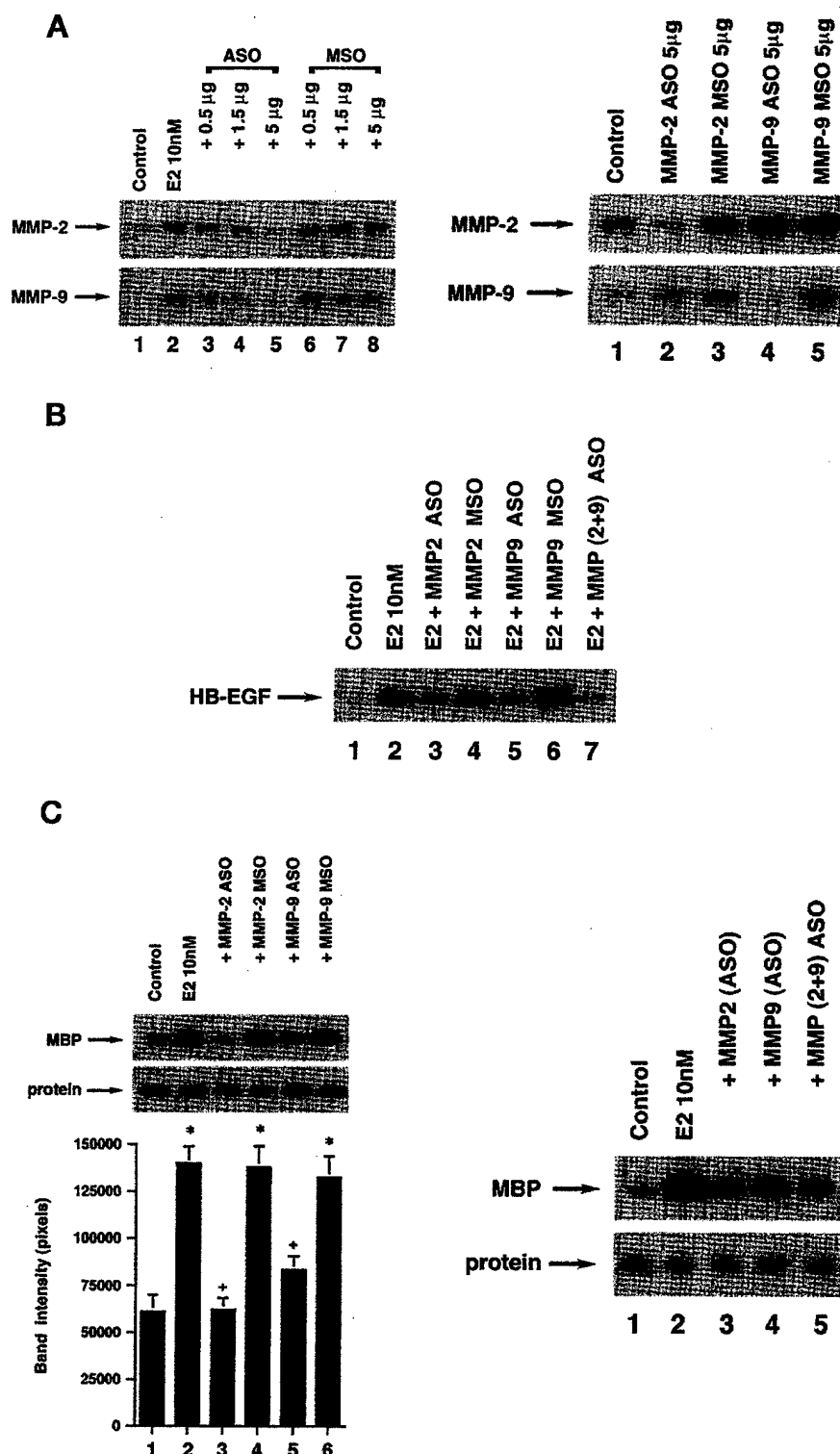


FIG. 4. Matrix metalloproteinases 2 and 9 mediate E2/ER effects. A, validation of ASO to MMP-2 and MMP-9. MCF-7 cells were incubated with ASO or MSO with LipofectAMINE, and MMP-2 or MMP-9 protein was detected by Western blot, 24 h after transfection. The left panel shows the dose-responsive effects of the ASO to MMP-2 (top panel) or to MMP-9 (bottom panel) to inhibit the intended protein production, but no effect of MSO was found. The right panel shows the specificity of an ASO for only its target MMP. Control is in the presence of E2. B and C, HB-EGF shedding (B) or ERK activation (C) in response to E2 is prevented by an MMP-2 or MMP-9 ASO but not MSO. HB-EGF shedding was carried out in MCF-7 cells, whereas ERK activation was determined in MCF-7 (left panel) or EC (right panel). The data are representative of three experiments, except for the bar graph, where three experiments are combined. *, $p < 0.05$ for control versus E2; +, $p < 0.05$ for E2 versus E2 with MMP-2 or MMP-9 ASO.

in the absence) of ER α , E2 stimulated the activation of MMP-2 and MMP-9 (Fig. 6C). This was partially dependent upon extracellular calcium, PLC, and PKC signaling. These data strongly support the idea that the classical ER α is required for

E2 to activate rapid signaling in breast cancer.

The Role of EGFR in E2 Activation of Multiple Signaling Pathways—Most studies invoking the role of EGFR in GPCR signaling have examined ERK activation. Regarding E2 signal-

incubated with E2 with or without PP2 (soluble Src inhibitor) or with E2 in cells transfected to express a dominant negative Src (pRC-csrc-K298M). By gelatin zymography (see "Experimental Procedures"), active (act) MMP-9 and MMP-2 are shown, along with the inactive (pro) MMP-2 protein. M.W., molecular weight. C, matrix metalloproteinase or ER inhibition prevents E2-induced ERK activation. MCF-7 cells (left panel) and ZR-75-1 cells (right panel) were incubated with E2 $\pm$ ICI182780 or GM6001 (MMPi), and ERK activity was determined. EGF was also added as a control. The bar graph is three experiments combined. *, $p < 0.05$ for control versus E2 or EGF; +, $p < 0.05$ for E2 versus E2 with MMPi. MMPi, matrix metalloproteinase inhibitor; MBP, myelin basic protein.

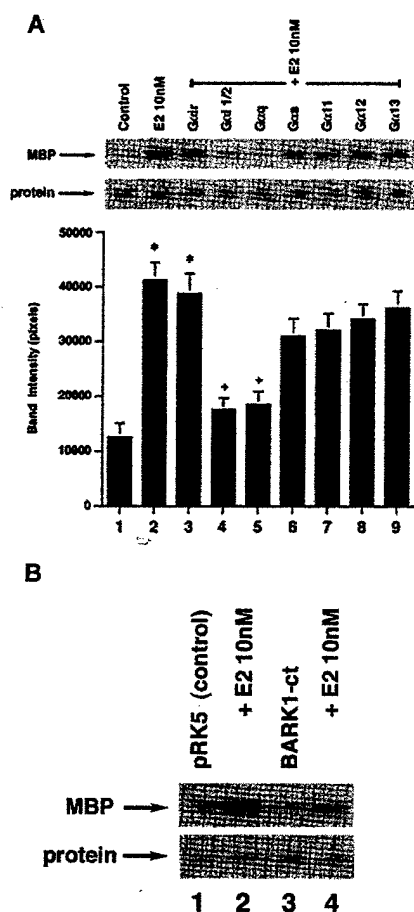


FIG. 5. $G\alpha$ subunit protein activation is required for E2-induced ERK activation in MCF-7 cells. A, expression of dominant negative mini-genes for $G\alpha_q$ and $G\alpha_i$ but not $G\alpha_{12}$, or $G\alpha_{13}$ prevents E2-induced ERK. The cells were transfected to express truncated $G\alpha$ subunits, serving as dominant negatives, then recovered, and exposed to E2 10 nM for 8 min. ERK activity was determined. Lanes 1 and 2 are nontransfected cells; lane 3 is E2-stimulated ERK after control plasmid transfection. The bar graph represents three experiments combined. *, $p < 0.05$ for control versus E2, or control versus E2-incubated, $G\alpha_{i2}$ -transfected cells. +, $p < 0.05$ for E2-incubated, $G\alpha_{i1}$ -transfected cells versus E2 in $G\alpha_q$ or $G\alpha_{13}$ mini-gene expressing cells. B, $G\beta\gamma$ contributes to E2-induced ERK activation. The cells were transiently transfected to express a dominant negative, C-terminally truncated β -adrenergic receptor kinase (BARK1-CT), the cells were recovered for 24 h, and then ERK activation by E2 was determined. A representative experiment, repeated twice, is shown.

ing, ERK and cAMP generation are the two pathways that have been identified to require EGFR activation (24, 25), but this has only been established in breast cancer cells. To further define the role of EGFR in E2-induced signaling from membrane ER, we examined breast cancer cells and EC, cells that express endogenous ER (9, 20). In MCF-7 cells, we found that E2 activated protein kinase B (AKT) (Fig. 7A). This was substantially prevented by the soluble inhibitor of MMP activity and by tyrphostin AG1478, implicating the EGFR. These two compounds had no effects by themselves (data not shown). In EC, we previously showed that E2 activates the p38 β member of the MAP kinase family, and this was essential for E2 to act as a cell survival factor during hypoxia, to preserve EC morphology after metabolic insult, and to stimulate EC migration and primitive capillary formation (33). Here, we show that MMP inhibition or EGFR tyrosine kinase inhibition significantly prevents E2 signaling to the activation of p38 β (Fig. 7B). Thus, additional signal transduction pathways are rapidly triggered by E2 in several cell types, and these pathways require

EGFR transactivation via the linked events we show here.

E Domain Activation of Signaling—What structural aspect of the membrane ER is necessary for activation of the signal cascade that results in EGFR activation and ERK up-regulation? This is an important issue, and assuming that the membrane and nuclear proteins are the same (6), there is no typical catalytic or kinase domain sequence present in ER α or ER β . It has recently been shown in CHO cells lacking endogenous ER that targeting of the E domain of ER α to the plasma membrane is sufficient to allow strong activation of ERK by E2 (5). This same, localized construct rescues HeLa cells from apoptotic cell death in response to etoposide (17), and in both situations, targeting of the E domain to the nucleus had no effect. We therefore asked whether targeting the E domain of ER α to the plasma membrane was sufficient to activate the signal pathway that we define here. This was accomplished in the HCC-1569 breast cancer cells that do not express ER. Targeting the E domain to the plasma membrane resulted in MMP activation (Fig. 8A) and EGFR activation (Fig. 8B), leading to ERK up-regulation (5). In the absence of the expressed E domain, E2 was unable to stimulate any of these events. Targeting the E domain to the nucleus also did not result in activation of this pathway. Therefore, the E domain appears to be sufficient for the complex interactions at the plasma membrane that allow assembly and activation of the signalsome in response to E2.

DISCUSSION

The ability of E2 to signal from membrane ER is increasingly appreciated as being important to the effects of this sex steroid. E2 triggers calcium increases in seconds and rapidly activates PKC and adenylate cyclase. Downstream activation of several kinases then leads to cell biological effects in a variety of cell types (44). Ischemia reperfusion injury of muscle in rats is limited by E2, and this occurs through the physical association of ER with phosphatidylinositol 3-kinase, the subsequent up-regulation of kinase activity, and the generation of NO (13). E2 acts as a survival factor for neurons (27), breast cancer (16, 45), and osteoblasts (17) while suppressing osteoclast differentiation (14). The sex steroid also acts as a survival and angiogenesis-promoting factor for EC (33). These effects are related to the modulation of ERK, JNK, and p38 MAP kinases, regulated through membrane ER. Recently, it was shown that the administration of antibodies to ER α in nude mice blocked the growth of human breast cancer xenografts. This probably resulted from the antibodies inhibiting membrane ER signaling to ERK and phosphatidylinositol 3-kinase (46). Therefore, it is important to understand how E2 acts through membrane ER to trigger signal transduction.

Now established for a variety of GPCRs, membrane ER localize substantially to caveolae (3–5). Here, they can physically complex with or activate signal molecules, including G proteins, receptor tyrosine kinases (insulin-like growth factor-1 receptor and EGFR), nonreceptor tyrosine kinases (Src family), and a variety of adapter and threonine/serine kinase proteins. This probably occurs on a scaffold platform provided by caveolin-1 (47, 48) and in part related to tyrosine 14 phosphorylation of this protein (49). Interestingly, this tyrosine is the principal substrate for Src kinase action (49), and the ability of E2 to activate Src at the membrane (8, 17) may therefore contribute to assembling the mature signalsome upon ER ligation by E2. Caveolin-1 can bind to and promote the assemblage of G proteins, Src, Grb7, Raf, Ras, MEK, and the EGFR at the plasma membrane (48). Caveolin-1 also facilitates ER translocation to the plasma membrane and localization within the caveolae microdomain (5). In this way, localizing ER and the signaling molecules to a confined area could augment the ability of E2/ER to transactivate EGFR, resulting in the stimulation of

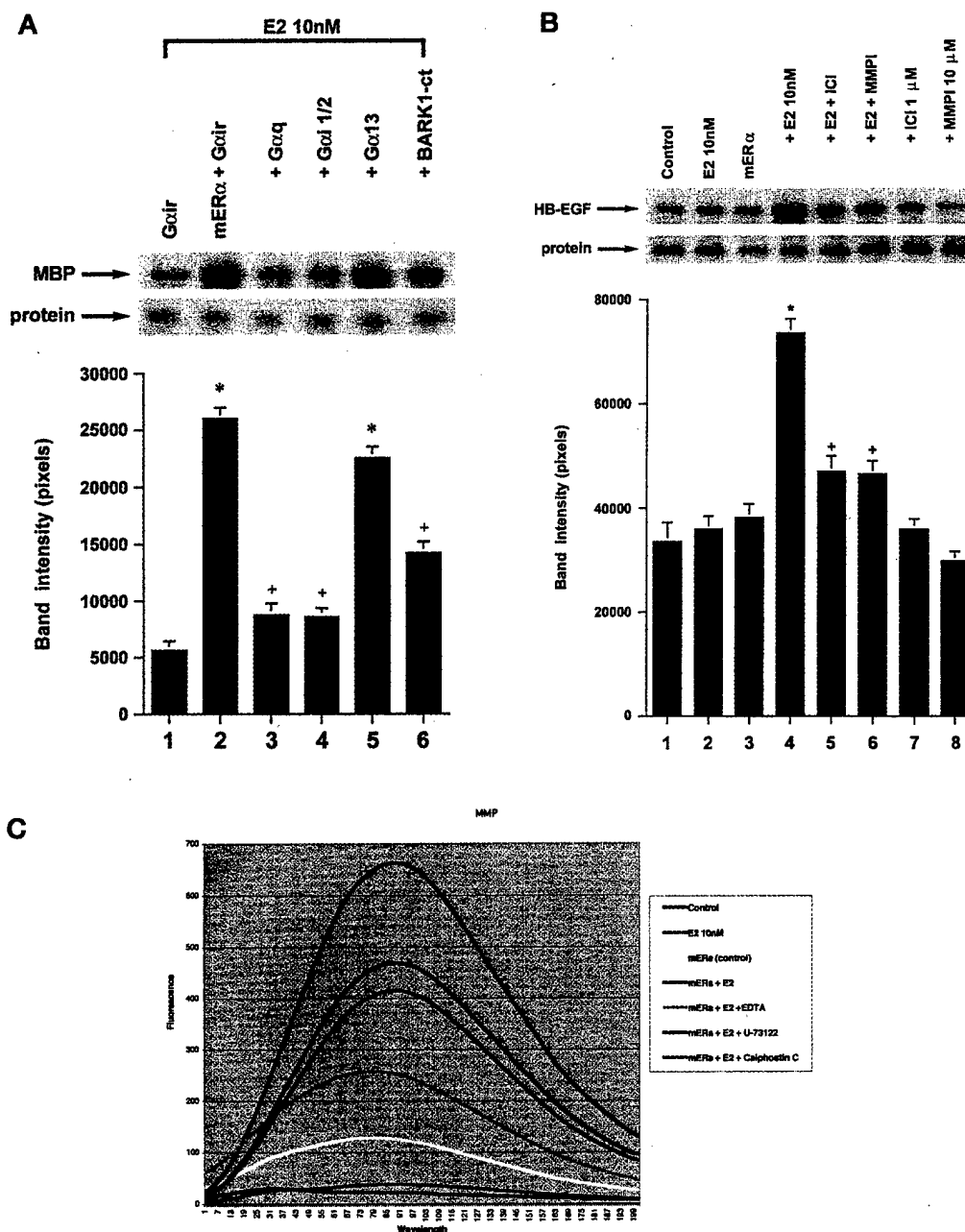


FIG. 6. ER is necessary for E2-induced proximal signaling. A, specific G protein subunits are required for ER-induced ERK. HCC-1569 cells were co-transfected to express ER α or pcDNA3 and truncated G α subunits or the C-terminal truncated β -adrenergic receptor kinase (BARK1-CT). The cells were recovered and then incubated with 10nME2 for 10 min, and ERK activity was determined. The bar graph represents two experiments combined. B, HB-EGF secretion in response to E2. ER α or pcDNA3 was expressed, and the cells were incubated with E2 with or without MMPI or ICI182780 for 3 min. Electrophoresed proteins were then subjected to Western blot. The bar graph is three experiments combined. C, MMPI activation by E2 requires ER. ER α -transfected HCC-1569 cells were incubated with E2 for 2 min, and the cell lysate was used to determine MMP activity by spectrofluometry. EDTA is a calcium chelator, U-73122 is a PLC inhibitor, and calphostin C is a PKC inhibitor.

ERK activity (this work and Ref. 25). However, upon GPCR ligation, caveolin probably dissociates from binding to the EGFR, leading to the activation of this receptor tyrosine kinase (50).

Although some details of the mechanisms of EGFR transactivation by ER (or any GPCR) are known, there are several aspects that are not clear. We found that the ability of membrane ER to activate G α_q and G α_i , but not the α subunits of G α_s , G α_{12} , and G α_{13} , was important to the subsequent (but still rapid) signaling events upstream of EGFR activation (Fig. 9). G $\beta\gamma$ inhibition also prevented the full transactivation of EGFR and ERK up-regulation in response to E2. This underscores the ideas that E2 activates several G proteins (6) and that there are

specific functions for each but with some degree of redundancy. The partial redundancy we demonstrate may be related to the requirement that full signaling by E2 requires multiple G protein activations. Supporting this idea, we found that co-expressing dominant negative mini-genes for G α_q and G α_i (but not co-expression with G α_s) added to the inhibition of E2/ER signaling to MMP activation and EGFR activation, compared with the inhibition of single G α subunits.² This may be related to the necessity for complete activation of Src and Src-induced signaling to MMP activation (see below).

² M. Razandi, A. Pedram, and E. R. Levin, unpublished observations.

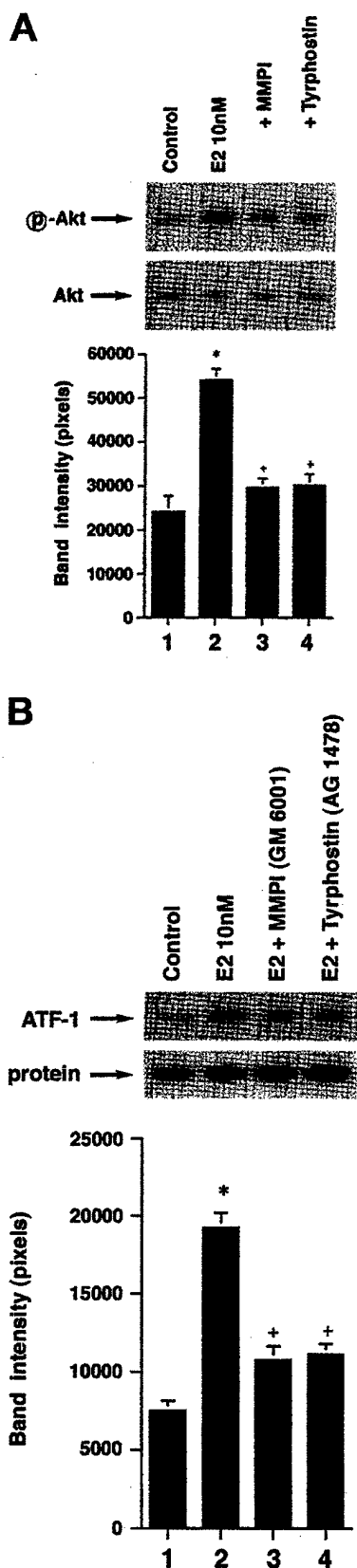


FIG. 7. Additional signaling pathways that depend upon ER to EGFR cross-talk. A, E2 induces AKT activation in MCF-7 cells, dependent upon MMP activation and EGFR tyrosine kinase function. MCF-7 cells were incubated with E2 with or without GM6001 or tyrophostin AG1478 for 10 min, and AKT phosphorylation at serine 473 was determined. B, p38 β activation in endothelial cells by E2 is significantly prevented by inhibition of MMP activity or the EGFR tyrosine kinase. EC were incubated with 10 nM E2 for 15 min, and the p38 immunopre-

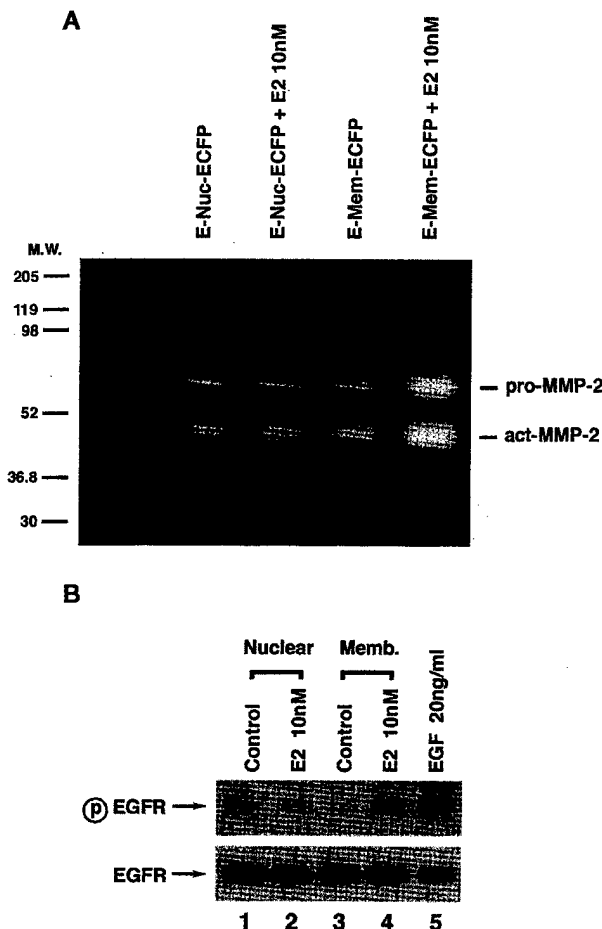


FIG. 8. Structure/function relationship between ER and signaling. A, targeting the E domain of ER α to the membrane of breast cancer cells augments MMP activity. HCC-1569 cells were transiently transfected to express the E domain targeted to the nucleus (lanes 1 and 2) or to the plasma membrane (lanes 3 and 4), followed by 2 min of treatment with E2 and determination of MMP activity. A representative study is shown, repeated once. B, expressing the E domain in the membrane leads to the transactivation of EGFR by E2. The transfected cells were assayed for EGFR phosphorylation by Western Blot, using an antibody against tyrosine 1138 (lane 3 versus lane 4). EGF-induced transactivation of its receptor serves as a positive control. The study was repeated twice.

We also found that MMP-2 and MMP-9 were necessary for E2 to stimulate the secretion of HB-EGF and the transactivation of EGFR. First, E2 activated these two enzymes, as determined by gelatin zymography and substrate cleavage studies. However, E2 did not up-regulate MMP-3 and MMP-13 activity, thus showing the specificity of our results. Second, E2 induced the release of HB-EGF into the cell culture medium after only 3 min of incubation, and this was substantially prevented by the specific antisense (but not missense) constructs for MMP-2 and MMP-9, with the effects being additive. Shedding of HB-EGF is a complicated process, and involvement of Ras-Raf-Mek (51), Rac (52), or PKC δ and the metalloprotease-disintegrin, MDC9 (37), has been proposed. In some cellular contexts, unknown metalloproteinase(s) mediates this shedding (53). Recently, TACE/ADAM17 has been shown to cleave expressed HB-EGF at 24 h in fibroblasts (54). However, our results suggest that MMP-2 and MMP-9 are sufficient.

cipitated from the cell lysate(s) was used for *in vitro* kinase activity assays, with ATF-1 as substrate. The bar graphs are from three experiments combined.

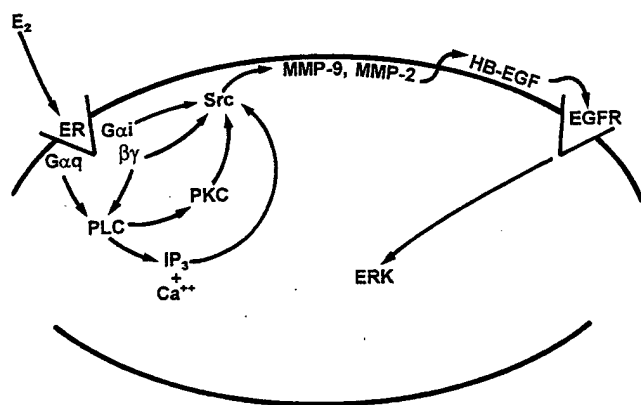


FIG. 9. E2-induced proximal signaling to the transactivation of EGFR, leading to ERK activation.

The specific signal from GPCRs that leads to MMP activation is not well understood. In this regard, we report the novel finding that Src is necessary for E2 to activate MMP-2 and MMP-9 and subsequent HB-EGF shedding. A previous study implicated Src as upstream from HB-EGF, but its role was undetermined (29). The precise mechanisms by which Src accomplishes MMP secretion and activation is unknown but is under investigation. It should be appreciated that this kinase is also *downstream* of EGFR, either through Src binding this receptor or through potential cross-talk of EGFR to G protein-coupled receptors, leading to Src activation (55, 56).

Our novel identification of MMP-2 and MMP-9 secretion and activation as being involved in estrogen signaling underlies the overall contribution of these MMPs to breast cancer biology. MMP-2 and MMP-9 have been implicated in the aggressive behavior of breast cancer (57, 58). The ability of breast cancer cells to migrate or invade/metastasize is importantly dependent on the degradation of cell matrix by MMPs. However, these proteases also play additional important roles to mediate cell survival, differentiation, and angiogenesis (reviewed in Ref. 59). Recently, MMP-2 production in response to E2 was found to be dependent on ERK signaling to the up-regulated activity of the AP-2 transcription factor in mesangial cells (60). In our model, MMP activation is necessary for E2 to stimulate HB-EGF secretion into the culture media, and HB-EGF but not TGF α transactivates the EGFR to signal to ERK.

The ability of EGFR to underlie E2/ER-induced ERK may represent only a single example of the wider signaling interactions of these two growth modulatory systems. We therefore asked whether other important signaling pathways that originate from membrane ER are also dependent on EGFR. We report here that E2 activates protein kinase B in breast cancer cells and p38 β MAP kinase in EC and that both pathways are also dependent upon transactivation of the EGFR. Utilization of EGFR to activate ERK is relatively common for a variety of GPCRs (38, 61); however, GPCRs can also activate ERK by pathways apart from EGFR (62). In this respect, we previously showed that E2 stimulates G α_s and cAMP, as well as ERK in CHO cells that are transiently transfected to express ER but that lack endogenous EGFR (6). Other EGF receptor family members might facilitate GPCR signaling to distinct pathways and thereby contribute to the specificity of signaling. For instance, the ability of muscarinic receptors to activate AKT is dependent upon the transactivation of the ErbB3 member of the EGFR family (63). In other situations, platelet-derived growth factor or insulin-like growth factor-1 receptors may be necessary for

GPCR effects (64–66). Thus, the tyrosine kinase receptor milieu in a particular cell may specifically control the panoply of signaling typically enacted by a GPCR ligand.

These interactions extend to cross-talk in both directions, including from the growth factor receptor tyrosine kinase to ER (67). Insulin-like growth factor-1 and EGF can signal to transcription via ER, independently of E2 (68, 69). This occurs through growth factor receptor-induced phosphorylation of the nuclear sex steroid receptor (70) or co-accessory proteins (71), and the induction of several kinase cascades is important in this regard (70, 72). These complex interactions are important in that they may contribute to the ability of breast cancer cells to proliferate or survive via ER, even when circulating levels of E2 are low, as in the post-menopausal woman.

An additional important issue is whether ER is required for E2 to activate signaling pathways from the membrane. It has previously been shown that E2 can transactivate the EGFR and signal in breast cancer cells that do not express ER (24). This purportedly occurs through a nondefined interaction with the orphan GPCR, GPR 30, and can nonspecifically be activated by estrogen receptor antagonists (ICI182730) and relatively inactive stereoisomers (17- α -E2). We report here that in the absence of ER, E2 can not activate ERK in HCC-1569 cells that lack this receptor. Expressing ER (or the E domain targeted to the cell membrane) allows E2 to signal through specific G proteins, MMP activation, and HB-EGF secretion that activates EGFR. Although some cells have been reported to respond rapidly to E2 in a nontraditional ER-related or ER-independent fashion (10, 73), the mechanisms underlying these reports remain unknown. Furthermore, the majority of studies indicate the requirement of ER for E2 action (5, 6, 8, 20, 27, 28), and these studies identify typical receptor pharmacology for the nongenomic actions of this sex steroid (reviewed in Ref. 43).

What part of ER is necessary for signal transduction at the membrane? Tyrosine 537 in the AF-2 portion of the E domain is essential for the interaction of ER with Src and functional up-regulation of ERK in MCF-7 cells (8). Recently, an interaction between the AF-1 domain of ER α and the phosphotyrosine binding and SH2 domains of the adapter protein Shc was postulated to mediate ERK activation in MCF-7 cells (20). However, Razandi *et al.* (5) recently showed that targeting the E domain of ER α to the plasma membrane of CHO cells is sufficient for robust ERK activation by E2, and Kousteni *et al.* (17) showed that this was sufficient to rescue HeLa cells from apoptosis. Here, we show that targeting the E domain to the membrane (and not to the nucleus) of HCC-1569 cells results in MMP-2 activation, EGFR transactivation, and ERK up-regulation. Thus, it is not clear what the significance of the AF-1 region of ER might be for signaling from the membrane. We propose that elements in the E domain, such as AF-2, allows for the complex interactions with G proteins, caveolin, Src, and other signaling molecules.

In summary, E2 activation of ERK is dependent on several G α and G $\beta\gamma$ subunits of small GTP-binding proteins. Src-dependent stimulation of MMP-2 and MMP-9 activity in response to E2/ER releases HB-EGF, leading to EGFR transactivation, and signaling to MAP kinase. The E domain of ER α appears to be sufficient to activate these mechanisms. The assemblage of signal transduction complexes probably platformed on caveolin or growth factor receptor tyrosine kinase proteins (EGFR and insulin-like growth factor receptor) accounts for much of the ability of E2 to signal through membrane-localized ER to different pathways. This mechanism is increasingly appreciated to play important roles in the cellular biology of E2 actions, and manipulation of these

pathways could therapeutically modulate the effects of this sex steroid.

REFERENCES

1. Tsai, M.-J., and O'Malley, B. W. (1994) *Annu. Rev. Biochem.* **63**, 451-486
2. Falkenstein, E., and Wehling, M. (2000) *Eur. J. Clin. Invest.* **3**, (suppl.) 51-54
3. Kim, H. P., Lee, J. Y., Jeong, J. K., Bae, S. W., Lee, H. K., and Jo, I. (1999) *Biochem. Biophys. Res. Commun.* **263**, 257-262
4. Chambliss, K. L., Yuhanna, I. S., Mineo, C., Liu, P., German, Z., Sherman, T. S., Mendelsohn, M. E., Anderson, R. G. W., and Shaul, P. W. (2000) *Circ. Res.* **87**, e44-e52
5. Razandi, M., Oh, P., Pedram, A., Schnitzer, J., and Levin, E. R. (2002) *Mol. Endocrinol.* **16**, 100-115
6. Razandi, M., Pedram, A., Greene, G. L., and Levin, E. R. (1999) *Mol. Endocrinol.* **13**, 307-319
7. Wyckoff, M. H., Chambliss, K. L., Mineo, C., Yuhanna, I. S., Mendelsohn, M. E., Mumby, S. M., and Shaul, P. W. (2001) *J. Biol. Chem.* **276**, 27071-27076
8. Migliaccio, A., Castoria, G., Di Domenico, M., de Falco, A., Bilancio, A., Lombardi, M., Vitorria Barone, M., Ametrano, D., Zannini, M. S., Abbondanza, C., Bontempo, P., and Auricchio, F. (2000) *EMBO J.* **19**, 5406-5417
9. Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E., and Auricchio, F. (1996) *EMBO J.* **15**, 1292-1300
10. Singh, M., Setalo, G., Jr., Guan, X., Warren, M., and Toran-Allerand, C. D. (1999) *J. Neurosci.* **19**, 1179-1188
11. Le Mellay, V., Grosse, B., and Lieberherr, M. (1997) *J. Biol. Chem.* **272**, 11902-11907
12. Ansonoff, M. A., and Etgen, A. M. (1998) *Endocrinology* **139**, 3050-3056
13. Simoncini, T., Hafezi-Moghadam, A., Brazil, D. P., Ley, K., Chin, W. W., and Liao, J. K. (2000) *Nature* **407**, 538-541
14. Shevde, N. K., Bendixen, A. C., Dienger, K. M., and Pike, J. W. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 7829-7834
15. Morey, A. K., Pedram, A., Razandi, M., Prins, B. A., Hu, R.-M., Biesiada, E., and Levin, E. R. (1997) *Endocrinology* **138**, 3330-3339
16. Razandi, M., Pedram, A., and Levin, E. R. (2000) *Mol. Endocrinol.* **14**, 1434-1447
17. Kousteni, S., Bellido, T., Plotkin, L. I., O'Brien, C. A., Bodenner, D. L., Han, L., Han, K., DiGregorio, G. B., Katzenellenbogen, J. A., Katzenellenbogen, B. S., Roberson, P. K., Weinstein, R. S., Jilka, R. L., and Manolagas, S. C. (2001) *Cell* **104**, 719-730
18. Pappas, T. C., Gametchu, B., Yannariello-Brown, J., Collins, T. J., and Watson, C. S. (1995) *FASEB J.* **9**, 404-410
19. Norfleet, A. M., Thomas, M. L., Gametchu, B., and Watson, C. S. (1999) *Endocrinology* **140**, 3805-3814
20. Russell, K. S., Haynes, M. P., Sinha, D., Clerisme, E., and Bender, J. R. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 5930-5935
21. Song, R. X., McPherson, R. A., Adam, L., Bao, Y., Shupnik, M., Kumar, R., and Santen, R. J. (2002) *Mol. Endocrinol.* **16**, 116-127
22. Aronica, S. M., Kraus, W. L., and Katzenellenbogen, B. S. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 8517-8522
23. Lieberherr, M., Grosse, B., Kachkache, M., and Balsan, S. (1993) *J. Bone Min. Res.* **8**, 1365-1376
24. Filardo, E. J., Quinn, J. A., Frackelton, A. R., Jr., and Bland, K. I. (2002) *Mol. Endocrinol.* **16**, 70-84
25. Filardo, E. J., Quinn, J. A., Bland, K. I., and Frackelton, A. R. (2000) *Mol. Endocrinol.* **14**, 1649-1660
26. Luttrell, L. M., Hawes, B. E., van Biesen, T., Luttrell, D. K., Lansing, T. J., and Lefkowitz, R. J. (1996) *J. Biol. Chem.* **271**, 19443-19450
27. Singer, C. A., Figueroa-Masot, X. A., Batchelor, R. H., and Dorsa, D. M. (1999) *J. Neurosci.* **19**, 2455-2463
28. Zhu, Y., Bian, Z., Lu, P., Karas, R. H., Bao, L., Cox, D., Hodgins, J., Shaul, P. W., Thoren, P., Smithies, O., Gustafsson, J. A., and Mendelsohn, M. E. (2002) *Science* **295**, 505-508
29. Pierce, K. L., Tohgo, A., Ahn, S., Field, M. E., Luttrell, L. M., and Lefkowitz, R. J. (2001) *J. Biol. Chem.* **276**, 23155-23160
30. Pedram, A., Razandi, M., and Levin, E. R. (1998) *J. Biol. Chem.* **273**, 26722-26728
31. Couse, J. F., Curtis, S. W., Washburn, T. F., Lindzey, J., Golding, T. S., Lubahn, D. B., Smithies, O., and Korach, K. S. (1995) *Mol. Endocrinol.* **9**, 1441-1454
32. Redemann, A., Holzmann, B., von Ruden, T., Wagner, E. F., Schlessinger, J., and Ullrich, A. (1992) *Mol. Cell. Biol.* **12**, 491-498
33. Razandi, M., Pedram, A., and Levin, E. R. (2000) *J. Biol. Chem.* **275**, 38540-38546
34. Koch, W. J., Hawes, B. E., Inglese, J., Luttrell, L. M., and Lefkowitz, R. J. (1994) *J. Biol. Chem.* **269**, 6193-6197
35. Gilchrist, A., Li, A., and Hamm, H. E. (2002) *Methods Enzymol.* **344**, 58-69
36. Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., and Ullrich, A. (1999) *Nature* **402**, 884-888
37. Izumi, Y., Hirata, M., Hasuwa, H., Iwamoto, R., Umata, T., Miyado, K., Tamai, Y., Kurisaki, T., Sehara-Fujisawa, A., Ohno, S., and Mekada, E. (1998) *EMBO J.* **17**, 7260-7272
38. Duffy, M. J., Maguire, T. M., Hill, A., McDermott, E., and O'Higgins, N. (2000) *Breast Cancer Res.* **2**, 252-257
39. Hua, J., and Muschel, R. J. (1996) *Cancer Res.* **56**, 5279-5284
40. Perret, S., Dockery, P., and Harvey, B. J. (2001) *Mol. Cell. Endocrinol.* **176**, 77-84
41. Rubio-Gayosso, I., Sierra-Ramirez, A., Garcia-Vazquez, A., Martinez-Martinez, A., Munoz-Garcia, O., Morato, T., and Ceballos-Reyes, G. (2000) *J. Cardiovasc. Pharmacol.* **36**, 196-202
42. Kelly, M. J., Wagner, E. J. (1999) *Trends Endocrinol. Metab.* **10**, 369-374
43. Sylvia, V. L., Walton, J., Lopez, D., Dean, D. D., Boyan, B. D., and Schwartz, Z. (2001) *J. Cell. Biochem.* **81**, 413-429
44. Levin, E. R. (2001) *J. Applied Physiol.* **91**, 1860-1867
45. Teixeira, C., Reed, J. C., and Pratt, M. A. (1995) *Cancer Res.* **55**, 3902-3907
46. Marquez, D. C., and Pietras, R. J. (2001) *Oncogene* **20**, 5420-5430
47. Liu, J., Oh, P., Horner, T., Rogers, R. A., and Schnitzer, J. E. (1997) *J. Biol. Chem.* **272**, 7211-7222
48. Okamoto, T., Schlegel, A., Scherer, P. E., and Lisanti, M. P. (1998) *J. Biol. Chem.* **273**, 5419-5422
49. Lee, H., Volonte, D., Galbiati, F., Iyengar, P., Lublin, D. M., Bregman, D. B., Wilson, M. T., Campos-Gonzalez, R., Bouzahzah, B., Pestell, R. G., Scherer, P. E., and Lisanti, M. P. (2000) *Mol. Endocrinol.* **14**, 1750-1775
50. Ushio-Fukaki, M., Hlenski, L., Santanam, N., Becker, P. L., Ma, Y., Griendling, K., and Alexander, R. W. (2001) *J. Biol. Chem.* **276**, 48269-48275
51. Gechtman, Z., Alonso, J. L., Raab, G., Ingber, D. E., and Klagsbrun, M. (1999) *J. Biol. Chem.* **274**, 18828-18835
52. Umata, T., Hirata, M., Takahashi, T., Ryu, F., Shida, S., Takahashi, Y., Tsuneoka, M., Miura, Y., Masuda, M., Horiguchi, Y., and Medada, E. (2001) *J. Biol. Chem.* **276**, 30475-30482
53. Dong, J., Opreko, L. K., Dempsey, P. J., Lauffenburger, D. A., Coffey, R. J., and Wiley, H. S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 6235-6240
54. Sunnarborg, S. W., Hinkle, C. L., Stevenson, M., Russell, W. E., Raska, C. S., Peschorn, J. J., Castner, B. J., Gerhart, M. J., Paxton, R. J., Black, R. A., and Lee, D. C. (2002) *J. Biol. Chem.* **277**, 12838-12845
55. Seta, K., Nanamori, M., Modrall, J. G., Neubig, R. R., and Sadoshima, J. (2002) *J. Biol. Chem.* **277**, 9268-9277
56. Luttrell, L. M., Daaka, Y., and Lefkowitz, R. J. (1999) *Curr. Opin. Cell Biol.* **11**, 177-183
57. Westermarck, J., and Kahari, V.-M. (1999) *FASEB J.* **13**, 781-792
58. Ray, J. M., and Stetler-Stevenson, W. G. (1994) *Eur. Respir. J.* **7**, 2062-2072
59. Coussens, L. M., Fingleton, B., and Matrisian, L. M. (2002) *Science* **295**, 2387-2392
60. Guccione, M., Silberberger, S., Lei, J., and Nuegarten, J. (2002) *Am. J. Physiol.* **282**, F164-F169
61. Hackel, P. O., Zwick, E., Prenzel, N., and Ullrich, A. (1999) *Curr. Opin. Cell Biol.* **11**, 184-189
62. Andreev, J., Galisteo, M. L., Kranenburg, O., Logan, S. K., Chiu, E. S., Okigaki, M., Cary, L. A., Moolenaar, W. H., and Schlessinger, J. (2001) *J. Biol. Chem.* **276**, 20130-20135
63. Tang, X., Batty, I. H., and Downes, C. P. (2002) *J. Biol. Chem.* **277**, 338-344
64. Schmidt, M., Frings, M., Mono, M. L., Guo, Y., Weernink, P. A., Evellin, S., Han, L., and Jakobs, K. H. (2000) *J. Biol. Chem.* **275**, 32603-32610
65. Kahlert, S., Nuedling, S., van Eickels, M., Vetter, H., Meyer, R., and Grohe, C. (2000) *J. Biol. Chem.* **275**, 18447-18453
66. Sumitomo, M., Milowsky, M. I., Shen, R., Navarro, D., Dai, J., Asano, T., Hayakawa, M., and Nanus, D. M. (2001) *Cancer Res.* **61**, 3294-3298
67. Yee, D., and Lee, A. V. (2000) *J. Mammary Gland Biol. Neoplasia* **5**, 107-115
68. Klotz, D. M., Curtis Hewitt, S., Ciana, P., Raviscioni, M., Lindzey, J. K., Foley, J., Maggi, A., DiAugustine, R. P., and Korach, K. S. (2002) *J. Biol. Chem.* **277**, 8531-8537
69. Curtis, S. W., Washburn, T., Sewall, C., DiAugustine, R., Lindzey, J., Couse, J. F., and Korach, K. S. (1996) *Proc. Natl. Acad. Sci.* **93**, 12626-12630
70. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. (1995) *Science* **270**, 1491-1494
71. Lopez, G. N., Turck, C. W., Schaufele, F., Stallcup, M. R., and Kushner, P. J. (2001) *J. Biol. Chem.* **276**, 22177-22182
72. Martin, M. B., Franke, T. F., Stoica, G. E., Chambon, P., Katzenellenbogen, B. S., Stoica, B. A., McLemore, M. S., Olivo, S. E., and Stoica, A. (2000) *Endocrinology* **141**, 4503-4511
73. Guo, Z., Krucken, J., Bente, W. P., and Wunderlich, F. (2002) *J. Biol. Chem.* **277**, 7044-7050

MINIREVIEW

Bidirectional Signaling between the Estrogen Receptor and the Epidermal Growth Factor Receptor

ELLIS R. LEVIN

Division of Endocrinology, Long Beach Veterans Affairs Medical Center, Long Beach, California 90822; and Departments of Medicine and Pharmacology, University of California, Irvine, California 92717

Interactions between the estrogen receptor (ER) and the epidermal growth factor receptor (EGFR) contribute to the biological effects of these binding protein families. EGFR stimulates DNA synthesis and gene transcription in the uterus, related in part to estrogen-independent activation of the nuclear ER. This results from signal transduction enacted by the plasma membrane tyrosine kinase growth factor receptor, leading to 1) phosphorylation and activation of the nuclear ER, and 2) phosphorylation of coregulator proteins. More recently, it has been shown that a pool of ER α resides in or associates with the plasma membrane as a cytoplasmic

protein. These ERs utilize the membrane EGFR to rapidly signal through various kinase cascades that influence both transcriptional and nontranscriptional actions of estrogen in breast cancer cells. This is congruent with a general theme of receptor signaling, where membrane G protein-coupled receptors activate tyrosine kinase growth factor receptors (EGFR, IGF-I receptor) that subsequently signal to MAPKs and other pathways. Overall, the bidirectional cross-talk between EGFR and cellular pools of ER contributes to reproductive organ physiology and pathophysiology. (*Molecular Endocrinology* 17: 309-317, 2003)

MOST GROWTH FACTORS activate cell proliferation, differentiation, or survival programs through binding their attendant tyrosine kinase receptors, expressed in the plasma membrane (1-4). As a result, the receptors undergo dimerization and conformational changes that result in transphosphorylation at discrete tyrosine residues. This provides binding sites for signaling or linker/adaptor molecules that contain Src-homology 2 domains, and the recruitment of additional signal molecules (5, 6). Such proteins include nonreceptor tyrosine kinases such as Src family members, or Grb and Sos family proteins. Signal cascades are then triggered, dependant upon the translocation, membrane association, and activation of tyrosine, serine/threonine, and lipid kinases, including ras, raf, protein kinase C, and phosphatidylinositol 3-kinase (PI3K). These kinases phosphorylate substrate proteins in the cytoplasm, altering target protein function. As an example, stimulation of PI3K results in AKT activation, which then phosphorylates a variety of proapoptotic proteins, including BAD, glycogen syn-

tase kinase-3 β , or Forkhead transcription factors (7, 8). This posttranslational modification sequesters/inactivates these proteins in cytoplasm, leading to cell survival.

Kinases also translocate to the nucleus, where they phosphorylate/activate and transcribe transcription factors that induce a variety of immediate early and late-arising genes. This important event underlies many of the biological effects of growth factor signaling. In fact, when nuclear localization of the ERK member of the MAPK family is prevented, cell proliferation often ceases (9). Important nuclear targets of ERK that are relevant to cell division include the transactivation of the cyclin D1 gene and the protooncogenes *c-fos* and *c-myc* (10-13). Therefore, the ability to signal from the membrane to both cytoplasmic and nuclear events is an essential feature of growth factor receptor function.

Steroid hormones have traditionally been conceived to act through the ligation of nuclear receptors (14). For estrogen, binding to estrogen receptor (ER) α or ER β results in an active complex in the nucleus that binds DNA directly at estrogen response elements within the promoters of target genes. Alternatively, estradiol (E₂)/ER promotes transcription factor binding to DNA (15). Liganded ER forms complexes with coregulator proteins (16), and constituents of the basal

Abbreviations: E₂, Estradiol; EGF, epidermal growth factor; EGFR, EGF receptor; ER, estrogen receptor; GPCR, G protein-coupled receptor; HB-EGF, heparin binding-EGF; IGF-IR, IGF-I receptor; KO, knockout; MMP, matrix metalloproteinase; PI3K, phosphatidylinositol 3-kinase; STAT, signal transducer and activator of transcription.

transcription machinery complex, leading to the modulation of RNA polymerase II activity, histone-induced chromatin unwinding, and transcription. However, it has become increasingly clear that estrogen (and other steroid hormones) also rapidly activates signaling in seconds to minutes, and this cannot be explained by any known function of nuclear receptors (17). Furthermore, ERs that lack a nuclear localization sequence (18) or truncated ERs that are targeted to the plasma membrane are fully capable of activating kinases and subsequent cell proliferation or survival (19, 20). A small pool of endogenous ERs that localize to the plasma membrane in various target cells can act similarly to classic growth factor receptors imbedded in the membrane. These ERs have been localized to caveolae raft domains isolated from the plasma membrane of target cells such as endothelial cells (19, 21). It is still unclear, however, whether these sex steroid receptors are integral membrane proteins and/or tether as cytoplasmic proteins to the cytoplasmic face of caveolae through binding to caveolin-1.

An important principle in the signaling field is that growth factor receptors cross-talk to each other. This includes heterodimerization between receptor family members, exemplified by the four members of the epidermal growth factor (EGF) receptor (EGFR) family (22). Additionally, signaling from one receptor activates cytoplasmic nonreceptor kinases (e.g., -Src) that positively or negatively modulate the activity of adjacent receptors (23). In this respect, EGFRs expressed on a population of cells may spread signal transduction enacted by a variety of unrelated growth factor receptors on adjacent cells (24).

Emerging data suggest cross-talk may exist between plasma membrane steroid receptors. Progesterone can stimulate ERK signaling via the utilization of ER (25), and estrogen or androgen can promiscuously stimulate signaling to ERK (and cell survival) through either sex steroid receptor (20). Furthermore, both membrane growth factor and steroid receptors interweave their actions with those of nuclear steroid receptors, thereby impacting cell biology. An example is that nuclear receptors transcribe genes, the protein products of which are acutely altered in function by phosphorylation, resulting from membrane receptor signaling.

In this overview, I will describe the current state of cross-talk between ERs and EGFRs. Work in this area has established a requirement of nuclear ER for some EGFR [and perhaps IGF-I receptor (IGF-IR)] actions. Recent findings suggest the important role of EGFR (or similar receptors) for estrogen signaling from the membrane in breast cancer. Bidirectional signaling between these essential cellular factors augments the actions of the individual steroid and growth protein.

SIGNALING FROM EGFRs TO NUCLEAR ERs

EGF binds to one or more members of the EGFR family that enact signaling cascades to the nucleus and cytoplasm, resulting in cell biological actions (22, 26). This pathway is indirectly used by E_2 . In reproductive organs, E_2 induces the EGFR and stimulates growth and rapid proteolytic activity in the uterus (27). Subsequent investigations established that this sex steroid stimulates the synthesis of EGF in this reproductive organ (28). Up-regulation of EGF probably explains the strong proliferative effect of E_2 on uterine epithelium, an action that is prevented by EGF antibody (29). Increased synthesis of EGF resulting from E_2 action extended the earlier observation that E_2 induces EGF secretion from breast cancer cells (30) and implicates this interaction in the growth of hormonally responsive cancer. In EGFR knockout (KO) mice, the stromal compartment, but not the epithelial response to E_2 , is severely limited in both the uterus and vagina (31).

A novel model of ER and EGFR interaction is derived from the observation that EGFR signaling depends upon an ER-mediated function but in an estrogen-independent fashion (32). Studies from Ignar-Trowbridge *et al.* (33) showed that EGF induction of DNA and lipid synthesis in the uterus could be prevented by ICI 164,384, an ER antagonist. More recent studies suggest that the effects of ER antagonists could be mediated through recruiting corepressors, thereby inhibiting growth factor-induced ER transcriptional effects (34). Continued work from the laboratories of DiAugustine and Korach (35) showed that EGF-induced DNA synthesis and transcription were absent in uteri from ER α KO (ERKO) mice. These results clearly show dependency on ER for EGF action in reproductive organs.

How does EGFR utilize ER for biological actions? Insight resulted from the observations that several peptide growth factor receptors signal to the phosphorylation and activation of the nuclear ER (36, 37). This includes EGF and was originally attributed to the ability of growth factor receptor-activated MAPK (ERK) to phosphorylate serine 118 in the A/B domain of the nuclear ER α . Serine 118 phosphorylation results in an increased ER-related transactivation of genes that are up-regulated by EGFR. Work by Ignar-Trowbridge *et al.* (38) showed that EGFR ligation induces the transcriptional up-regulation of an estrogen response element reporter construct, in ER-dependent fashion. This group also demonstrated that EGFR-to-ER cross-talk requires the A/B domain of ER α (39). Subsequent studies implicated several kinases that phosphorylate additional residues within ER α , resulting in the increased transcriptional activity of the nuclear receptor (40-44). Thus, impact of the growth factor receptor-ER interaction depends upon the signaling milieu within a particular cell that differentially phosphorylates numerous residues in the nuclear ER.

Another mechanism through which EGFR-induced signaling modulates ER transcriptional activity is via coregulator protein phosphorylation. As an example, EGF-induced ERK phosphorylates serine 736 of glucocorticoid receptor interacting protein 1. This increases the activity of this nuclear receptor nonspecific coactivator protein (45). EGF-triggered Src and Jnk activation may have a similar function for the cAMP response element-binding protein (46). Other coactivator proteins that are important and specific to ER function could be similarly activated or recruited through signaling-induced posttranslational modifications. Interestingly, growth factor receptors signal to cyclin D1 production, as part of promoting G₁/S phase cell cycling (47). Cyclin D1 activates ER transcriptional function (48) and interacts with the coactivator proteins, steroid receptor coactivator 1 and cAMP response element-binding protein/p300, as an additional mechanism to amplify nuclear ER action (49, 50).

It is conceivable that EGFR signaling also inhibits the activity/function of corepressor proteins on targeted promoters, and that other EGFR family members could also cross-talk to the nuclear ER. In breast cancer and other estrogen target cells, EGFR family members often heterodimerize, and ligands [heparin

binding-EGF (HB-EGF), TGF α , or EGF] can be somewhat promiscuous in their binding. Such considerations may be relevant to the interactions of the erb2/Neu oncogene and ER in early breast cancer. In this respect, breast tumor formation in mouse mammary tumor virus-erb2/neu mice is delayed on an ERKO background (51). A summary of mechanisms of EGFR signaling through ER is seen in Fig. 1.

Finally, it has recently been reported that the EGFR translocates to the nucleus, where it can bind to AT-rich DNA sequences and modulate the transcription of the cyclin D1 gene (52). Modulation of this controversial event by EGF occurs 48 h after ligation (53), and any interactions with the nuclear ER would be expected to impact the more chronic effects of the growth factor receptor.

In parallel to the interaction between the EGF system and ER, there is abundant evidence indicating cross-talk between the IGF-I system and ER. IGF-I binding activates its receptor, leading to PI3K/AKT activation, increased ER α synthesis, and augmented ER α transcriptional activity. This probably results from the phosphorylation of several serine residues in the AF-1 region (43) and EGFR accomplishes a similar action. Similarly to EGF, IGF-I activates parameters of

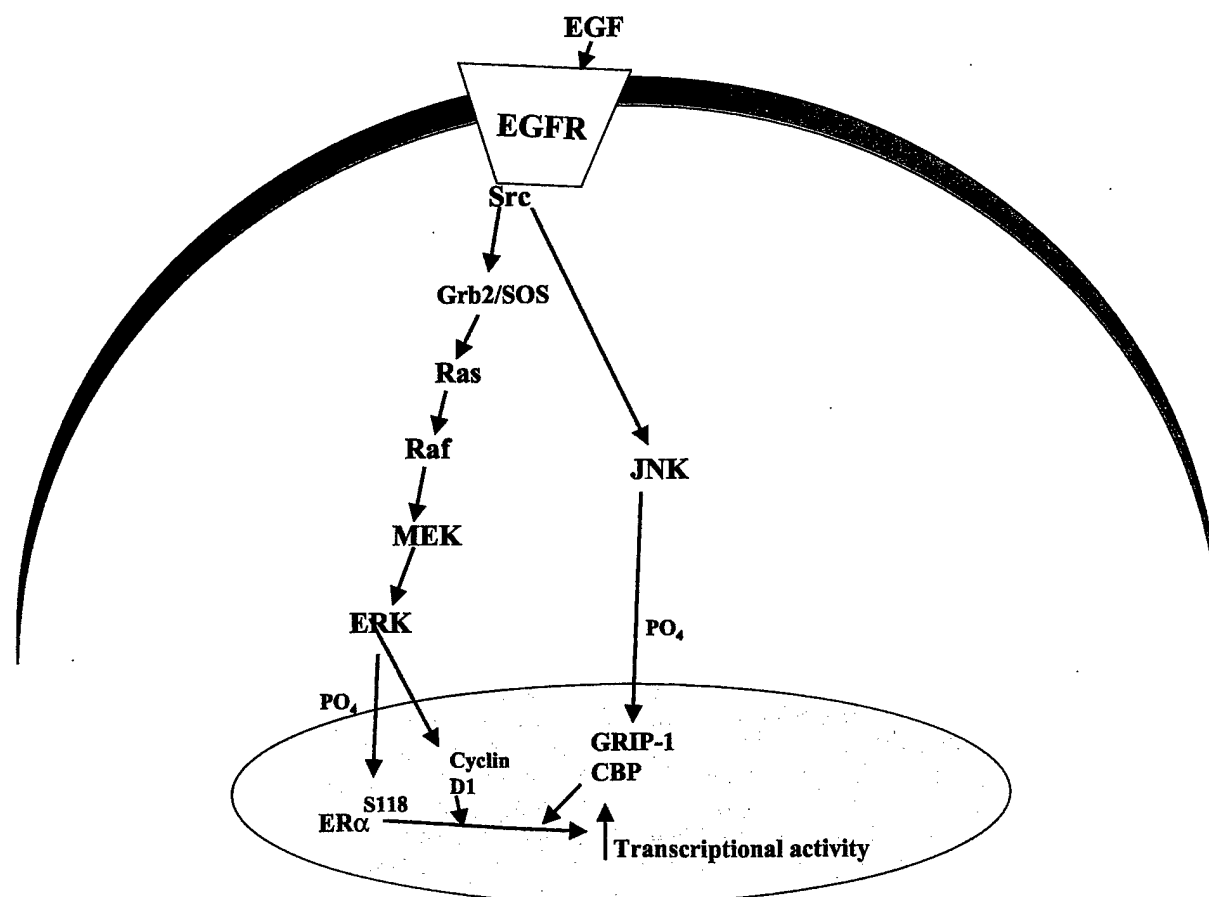


Fig. 1. EGFR Activation of ER or Coregulator Proteins via Signaling through MAPK Cascades
GRIP-1, Glucocorticoid receptor-interacting protein; CBP, cAMP response element-binding protein.

uterine cell proliferation *in vivo*, and this is dependent on ER α (54). Interestingly, in both the uterus and in breast cancer models, IGF-I signaling to ERK and PI3K/AKT is unaffected by ER α loss or antagonism (54, 55). When ER α is reexpressed in breast cancer cells that have lost ER through repetitive culturing, both E₂ and IGF-I resume their growth-inducing function (56). E₂ stimulates many proteins in the IGF-I system, including IRS proteins, IGF-IR, and IGF-binding proteins (57, 58), and ER α binds and phosphorylates the IGF-1R and enhances signaling through the growth factor receptor (59). In breast cancer, IGF-I and E₂ cooperate to promote G₁/S cell cycle progression (60, 61), and in the uterus of the IGF-I KO mouse, E₂-induced growth is absent (62). Thus, there appears to be an important cooperation and cross-talk between these two systems as well.

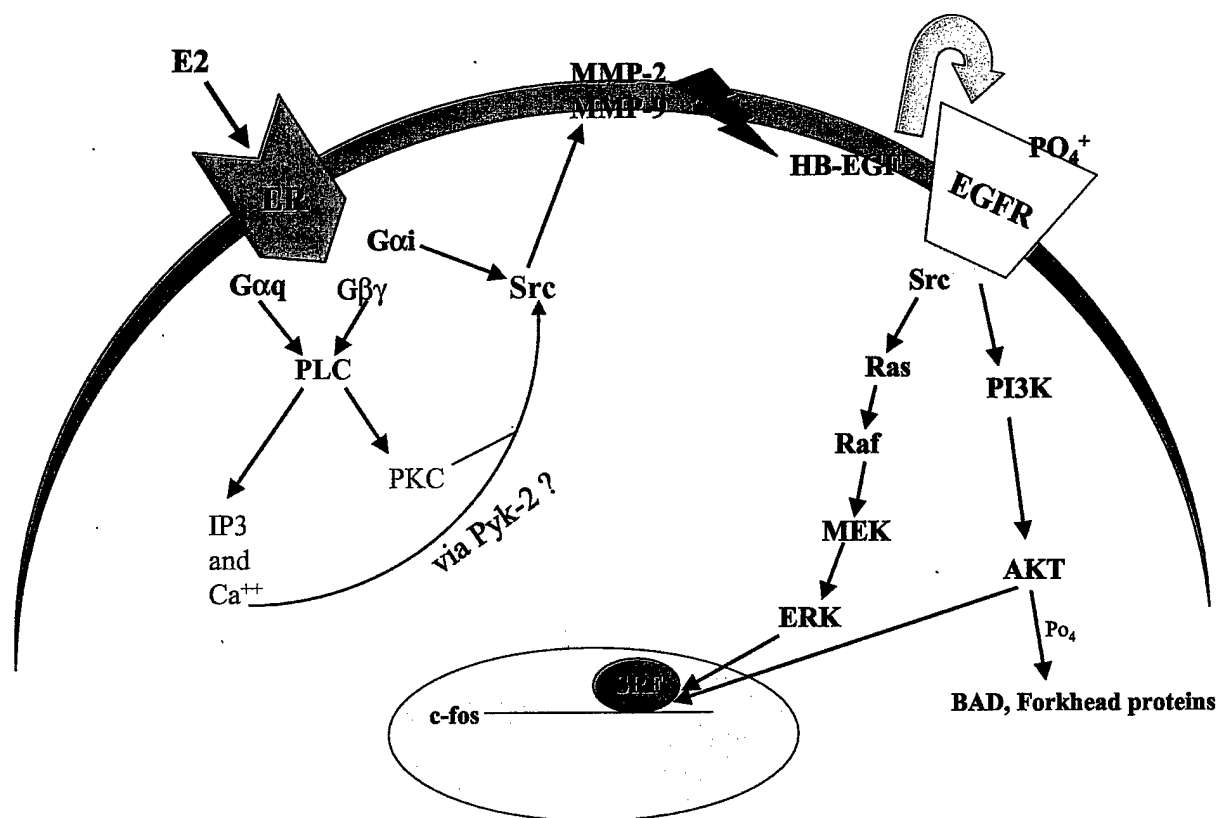
SIGNALING FROM ER THROUGH EGFR

The realization that E₂ has rapid effects in cells led to the characterization of the many generated signals. E₂ stimulates calcium channel opening and calcium influx or mobilization within seconds of binding receptors expressed in target tissues (63, 64). E₂ rapidly generates cAMP (65), phospholipase C, and inositol phosphate (66, 67). This results from G protein activation, and these early signals are transmitted to the rapid stimulation of protein kinase C, protein kinase A, MAPK, and PI3K (68). Functional and immunohistological identification of endogenous membrane ER (69, 70) led to the characterization of these receptors after expression of the cDNAs for classical ER α and ER β in Chinese hamster ovary cells (71). These latter studies indicated that membrane ER physically associate with and activate various G protein α -subunits, including G α s and G α q. G protein activation explains how ER generates cAMP (G α s function) or inositol 1,4,5-triphosphate and calcium (G α q function), as examples. Subsequent work showed that endogenous ER α activates G α i, leading to the generation of nitric oxide in endothelial cells (72).

An important finding described by Ullrich and colleagues (73) indicates that several G protein-coupled receptors (GPCRs) signal to ERK via the transactivation of the EGFR. Later studies from other laboratories confirmed and extended these observations to many GPCRs and provided additional details underlying this cross-talk. Identification of the membrane ER as a receptor capable of activating G proteins (71, 72) invoked the possibility that this receptor signaled through cross-talk/activation of the membrane EGFR. Filardo *et al.* (74, 75) showed that estrogen rapidly acts in breast cancer cells to stimulate the transactivation of EGFR, leading to cAMP and ERK up-regulation. This occurs through a linked path, first described for other GPCRs by Ullrich and colleagues (76). E₂ induces mainly unknown proximal signaling to cause the

activation of undefined matrix metalloproteinases (MMPs). Increased MMP function leads to the liberation of HB-EGF, which then binds and activates the EGFR. However, Filardo *et al.* (74) reported that 17 β -E₂, 17 α -E₂, or the ER antagonist, ICI 182780, were equivalent in activating EGFR and ERK. EGFR transactivation was proposed to occur independently of any ER and resulted from an undetermined effect of E₂ to activate the orphan GPCR, GPR 30 (77). More recent studies from Razandi *et al.* (78) demonstrated that E₂ requires an ER to signal to EGFR in breast cancer and is consistent with most studies that show an ER is necessary for rapid signaling by E₂ at the membrane (19, 42, 66, 79-81). Razandi *et al.* (78) also found that E₂/ER triggers a G α q, G α i, and G β γ -dependent activation of MMP-2 and -9, mediated through Src activation. By antisense studies, MMP-2 and MMP-9 were shown to be necessary for E₂-induced HB-EGF cleavage and liberation, the transactivation of EGFR, and downstream signaling to ERK and PI3K in breast cancer cells, and p38 MAPK in endothelial cells. It is possible that GPR30 may complex with and mediate membrane ER cross-talk to EGFR. However, recent studies from Ahola *et al.* (82) have called this idea into question. These investigators found that antisense inhibition of endogenous GPR-30 had no effect on E₂ signaling to cell proliferation in MCF-7 cells. Thus, this definitive approach suggests that GPR30 is not required and supports previous studies that ER 1) directly associates with and activates G proteins, and 2) this leads to downstream signaling (71, 72). The molecules involved in the ER-to-EGFR cross-talk are shown in Fig. 2.

The full extent of membrane-initiated signaling by E₂/ER and its dependence on EGFR remains to be defined, and the *in vivo* significance is incompletely understood. However, it was demonstrated more than 10 yr ago, that EGF antibody prevents E₂-induced vaginal and uterine growth (29), implying that cross-talk from ER to the EGFR at the membrane may be physiologically important. Recent studies concerning the role of E₂/ER signaling at the membrane support this idea. Simoncini *et al.* (83) showed that in endothelial cells, ER α directly associates with the membrane-tethered p85 subunit of PI3K. E₂ rapidly activates this kinase, leading to the generation of nitric oxide, and the rescue of rats from ischemia-reperfusion injury of their muscle. It is known that EGFR and PI3K associate (84), and so it is possible that a multi-protein complex exists between ER/PI3K/EGFR and endothelial nitric oxide synthase molecules, perhaps scaffolded onto caveolin-1 at the membrane (19, 21, 85). Similarly, Migliaccio *et al.* (79) showed that ER and Src form a complex. The interaction between ER and Src may be mediated by a newly described adapter protein, modulator of nongenomic activity of estrogen receptor (86). Src activation by E₂ leads to a kinase cascade resulting in ERK activation and DNA synthesis in cancer cells (79). Interestingly, EGFR and Src associate, and both molecules also form complexes



Cell proliferation and survival

Fig. 2. Membrane ER Cross-Talk to EGFR Leads to Downstream Signaling and Changes in Cell Biology of Breast Cancer

with caveolin-1 (87). Src or EGFR phosphorylates caveolin-1 at the important tyrosine 14, and this leads to the down-regulation of signaling (88). The ability of E₂/ER at the membrane to signal to ERK (via the demonstrated EGFR transactivation) has additional importance for cell biology. Song *et al.* (89) recently demonstrated that ER α lacking a nuclear localization signal and targeted to the plasma membrane activates ERK and cell proliferation in Chinese hamster ovary cells. Also, the survival of breast cancer cells that are subjected to radiation or taxol chemotherapy is enhanced by E₂, partially through ERK activation (90). In aggressive breast cancer, a truncated MTA1 protein was recently found to be highly expressed (91). This protein sequesters ER away from the nucleus and strongly reduces E₂-activated transcription, yet promotes increased ERK signaling and aggressive behavior of the tumor. In neurons subjected to several inducers of apoptosis, E₂ protects these cells through ERK activation (92). The actions of E₂ mediated by this MAPK occur through both protein phosphorylation (90) and gene transcription (93, 94). Most recently, bone loss *in vivo* was prevented by a compound (4-estren-3 α , 17 β -diol) that has no direct transcriptional activities but activates ERK signaling (95). Therefore, it is probable that the cross-talk from membrane ER through EGFR

to downstream kinase activation is biologically important.

The precise structural aspects of the membrane ER that are required for G protein activation are unclear at present but appear to mainly reside in the E domain. This conclusion is based upon the observations that targeting the E domain alone to the plasma membrane allows E₂ activation of ERK (19) and rescues bone cells from an apoptotic cell death (20). Similarly, sending the E domain to the plasma membrane of ER-negative, breast cancer cells results in E₂-induced, Src-dependent matrix metalloproteinase activation, HB-EGF liberation, and EGFR transactivation (78). Thus, the membrane E domain alone can recapitulate the key elements of the pathway from ER to EGFR. These findings are supported by the earlier observation that Src complexes with (and is activated by) E₂/ER, and that tyrosine 537 within the E domain is an essential structural component (96). This may be important for specific signaling pathways, however. Bjornstrom and Sjoberg (97) have recently examined the E₂ rapid activation of signal transducer and activator of transcription (STAT) transcription factor-induced β -casein promoter activation. STAT activation requires both ERK and PI3K, induced by E₂/ER. These authors report that mutating tyrosine 541 of the mouse ER α (equivalent of

human ER α tyrosine 537) has no effect on E₂ induction of the STAT- β -casein pathway. Also, Song *et al.* (89) recently showed that the Src homology 2-domain containing adapter protein, Shc, complexes with ER α through the AF-1 domain, and suggested that this interaction may underlie E₂-induced ERK. However, we recently found that expression of only the membrane-targeted E domain (19), or A/B domain-deleted ER α (unpublished observations) 1) fully binds steroid at the membrane, and 2) comparably activates ERK, compared with expressed wild-type ER α . Thus, current data support a unique and complete role for the E domain in effecting signal transduction initiated at the membrane.

PERSPECTIVE

The bidirectional cross-talk between ER and the growth factor receptors EGFR and IGF-IR indicates a potent method of augmenting E₂ or growth factor action. In a particular cell type and situation, there may be a predominant contribution from one of these pathways, essential to the cell biology of breast cancer, for instance. Tamoxifen is effective in preventing the reoccurrence of ER-positive breast cancer, in part because it inhibits aspects of E₂ and EGFR signaling. In ER-negative breast cancer, there is possibly less restraint on EGFR signaling to cell proliferation or survival in the absence of ER antagonism, thereby contributing to a more aggressive phenotype. Interestingly, in human breast cancer, ER and EGFR concentrations are inversely correlated (98, 99), and ER appears to repress the EGFR gene through a first intron sequence (98). Increased EGFRs in ER-negative breast cancer may also contribute to the more active growth and invasive behavior of these tumors.

The interactions of ER and EGFR impact both the transcriptional and nontranscriptional effects of steroid hormones and protein growth factors, but these are not mutually exclusive actions. Membrane E₂/ER activates PI3K signaling via EGFR (78). As shown by DNA microarray, PI3K activation by E₂ leads to the up-regulation of 250 genes after just 40 min of exposure of endothelial cells to sex steroid (100). Thus, ER-EGFR cross-talk at the membrane enacts multiple signaling pathways that likely have a profound impact on the transcriptional effects of E₂. It is certainly possible that manipulating the specific pathways that result from the bidirectional signaling will yield therapeutic interventions for human disorders that result from excessive growth factor and steroid hormone action.

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I regret that many fine contributions to this scientific area could not be recognized due to space limitations.

Address all correspondence and requests for reprints to: Ellis R. Levin, M.D., Medical Service (111-I), Long Beach Veterans Affairs Medical Center/University of California-Irvine, 5901 East 7th Street, Long Beach, California 90822. E-mail: ellis.levin@med.va.gov.

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REFERENCES

1. Wilkie AO, Patey SJ, Kan SH, Van Den Ouweland AM, Hamel BC 2002 FGFs, their receptors, and human limb malformations: clinical and molecular correlations. *Am J Med Genet* 112:266–278
2. Sledge Jr GW 2002 Vascular endothelial growth factor in breast cancer: biologic and therapeutic aspects. *Semin Oncol* 29(Suppl 11):104–110
3. Fant ME, Weisoly D 2001 Insulin and insulin-like growth factors in human development: implications for the perinatal period. *Semin Perinatol* 25:426–435
4. Betsholtz C, Karlsson L, Lindahl P 2001 Developmental roles of platelet-derived growth factors. *Bioessays* 23:494–507
5. Pawson T, Gish GD, Nash P 2001 SH2 domains, interaction modules and cellular wiring. *Trends Cell Biol* 11:504–511
6. Yaffe MB 2002 Phosphotyrosine-binding domains in signal transduction. *Nat Rev Mol Cell Biol* 3:177–186
7. Datta SR, Brunet A, Greenberg ME 1999 Cellular survival: a play in three Acts. *Genes Dev* 13:2905–2927
8. Brunet A, Datta SR, Greenberg ME 2001 Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. *Curr Opin Neurobiol* 11:297–305
9. Formstecher E, Ramos JW, Fauquet M, Calderwood DA, Hsieh JC, Canton B, Nguyen XT, Bamier JV, Camonis J, Ginsberg MH, Chneiweiss H 2001 PEA-15 mediates cytoplasmic sequestration of ERK MAP kinase. *Dev Cell* 1:239–250
10. Watanabe G, Lee RJ, Albanese C, Rainey WE, Batlle D, Pestell RG 1996 Angiotensin II activation of cyclin D1-dependent kinase activity. *J Biol Chem* 271:22570–22577
11. Weber JD, Raben DM, Phillips PJ, Baldassare JJ 1997 Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G1 phase. *Biochem J* 326:61–32668
12. Babu GJ, Lalli MJ, Sussman MA, Sadoshima J, Periasamy M 2000 Phosphorylation of elk-1 by MEK/ERK pathway is necessary for c-fos gene activation during cardiac myocyte hypertrophy. *J Mol Cell Cardiol* 32:1447–1457
13. Kerkhoff E, Houben R, Löffler S, Troppmair J, Lee JE, Rapp UR 1998 Regulation of c-myc expression by Ras/Raf signalling. *Oncogene* 16:211–216
14. White R and Parker MG 1998 Molecular mechanisms of steroid hormone action. *Endocr Relat Cancer* 5:1–14
15. Sukovich DA, Mukherjee R, Benfield PA 1994 A novel, cell-type-specific mechanism for estrogen receptor-mediated gene activation in the absence of an estrogen-responsive element. *Mol Cell Biol* 14:7134–7143
16. Halachmi S, Marden E, Martin G, MacKay H, Abbonanza C, Brown M 1994 Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. *Science* 264:1455–1458
17. Falkenstein E, Wehling M 2000 Nongenomically initiated steroid actions. *Eur J Clin Invest* 30:51–54

18. Zhang Z, Maier B, Santen RJ, Song RX 2002 Membrane association of estrogen receptor α mediates estrogen effect on MAPK activation. *Biochem Biophys Res Commun* 294:926–933
19. Razandi M, Oh P, Pedram A, Schnitzer J, Levin ER 2002 Estrogen receptors associate with and regulate the production of caveolin: implications for signaling and cellular actions. *Mol Endocrinol* 16:100–115
20. Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS, Roberson PK, Weinstein RS, Jilka RL, Manolagas SC 2001 Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell* 104:719–730
21. Chambliss KL, Yuhanna IS, Mineo C, Liu P, German Z, Sherman TS, Mendelsohn ME, Anderson RG, Shaul PW 2000 Estrogen receptor α and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae. *Circ Res* 87:e44–e52
22. Alroy I, Yarden Y 1997 The ErbB signaling network in embryogenesis and oncogenesis: signal diversification through combinatorial ligand-receptor interactions. *FEBS Lett* 410:83–86
23. Schlessinger J 2000 Cell signaling by receptor tyrosine kinases. *Cell* 103:211–225
24. Pierce KL, Tohgo A, Ahn S, Field ME, Luttrell LM, Lefkowitz RJ 2001 Epidermal growth factor (EGF) receptor-dependent ERK activation by G protein-coupled receptors: a co-culture system for identifying intermediates upstream and downstream of heparin-binding EGF shedding. *J Biol Chem* 276:23155–23160
25. Migliaccio A, Piccolo D, Castoria G, Di Domenico M, Bilancio A, Lombardi M, Gong W, Beato M, Auricchio F 1998 Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross talk with estrogen receptor. *EMBO J* 17:2008–2018
26. Riesell DJ, Stern DF 1998 Specificity within the EGF family/ErbB receptor family signaling networks. *Bioessays* 20:41–48
27. Mukku VR, Stancel GM 1985 Regulation of epidermal growth factor receptor by estrogen. *J Biol Chem* 260:9820–9824
28. DiAugustine RP, Petrusz P, Bell GI, Brown CF, Korach KS, McLachlan JA, Teng CT 1988 Influence of estrogens on mouse uterine epidermal growth factor precursor protein and messenger ribonucleic acid. *Endocrinology* 122:2355–2363
29. Nelson KG, Takahashi T, Bossert NL, Walmer DK, McLachlan JA 1991 Epidermal growth factor replaces estrogen in the stimulation of female genital-tract growth and differentiation. *Proc Natl Acad Sci USA* 88:21–25
30. Dickson RB, Huff KK, Spencer EM, Lippman ME 1986 Induction of epidermal growth factor-related polypeptides by 17 β -estradiol in MCF-7 human breast cancer cells. *Endocrinology* 118:138–142
31. Horn YK, Young P, Wiesen JF, Miettinen PJ, Derynck R, Werb Z, Cunha GR 1998 Uterine and vaginal organ growth requires epidermal growth factor receptor signaling from stroma. *Endocrinology* 139:913–921
32. Vignon F, Bouton MM, Rochefort H 1987 Antiestrogens inhibit the mitogenic effect of growth factors on breast cancer cells in the total absence of estrogens. *Biochem Biophys Res Commun* 146:1502–1508
33. Ignar-Trowbridge DM, Nelson KG, Bidwell MC, Curtis SW, Washburn TF, McLachlan JA, Korach KS 1992 Coupling of dual signaling pathways: epidermal growth factor action involves the estrogen receptor. *Proc Natl Acad Sci USA* 89:4658–4662
34. Huang HJ, Norris JD, McDonnell DP 2002 Identification of a negative regulatory surface within estrogen receptor α provides evidence in support of a role for corepressors in regulating cellular responses to agonists and antagonists. *Mol Endocrinol* 16:1778–1792
35. Curtis SW, Washburn T, Sewall C, DiAugustine R, Lindzey J, Couse JF, Korach KS 1996 Physiological coupling of growth factor and steroid receptor signaling pathways: estrogen receptor knockout mice lack estrogen-like response to epidermal growth factor. *Proc Natl Acad Sci USA* 93:12626–12630
36. Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige X, Gotoh Y, Nishida E, Kawashima H 1995 Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* 270:1491–1494
37. Bunone G, Briand PA, Miksicki RJ, Picard D 1996 Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J* 15:2174–2183
38. Ignar-Trowbridge DM, Teng CT, Ross KA, Parker MG, Korach KS, McLachlan JA 1993 Peptide growth factors elicit estrogen receptor-dependent transcriptional activation of an estrogen-responsive element. *Mol Endocrinol* 7:992–998
39. Ignar-Trowbridge DM, Pimentel M, Parker MG, McLachlan JA, Korach KS 1996 Peptide growth factor cross talk with the estrogen receptor requires the A/B domain and occurs independently of protein kinase C or estradiol. *Endocrinology* 137:1735–1744
40. Joel PB, Smith J, Sturgill TW, Fisher TL, Blenis J, Lannigan DA 1998 pp90rsk1 Regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. *Mol Cell Biol* 18:1978–1984
41. Chen D, Washbrook E, Sarwar N, Bates GJ, Pace PE, Thirunuvakkarasu V, Taylor J, Epstein RJ, Fuller-Pace FV, Egly JM, Coombes RC, Ali S 2002 Phosphorylation of human estrogen receptor α at serine 118 by two distinct signal transduction pathways revealed by phosphorylation-specific antisera. *Oncogene* 21:4921–4931
42. Lee H, Bai W 2002 Regulation of estrogen receptor nuclear export by ligand-induced and p38-mediated receptor phosphorylation. *Mol Cell Biol* 22:5835–5845
43. Martin MB, Franke TF, Stoica GE, Chambon P, Katzenellenbogen BS, Stoica BA, McLemore MS, Olivio SE, Stoica A 2000 A role for Akt in mediating the estrogenic functions of epidermal growth factor and insulin-like growth factor I. *Endocrinology* 141:4503–4511
44. Wang, R-A, Mazumdar, A, Vadlamudi RK, Kumar R 2002 P21-activated kinase-1 phosphorylates and transactivates estrogen receptor- α and promotes hyperplasia in mammary epithelium. *EMBO J* 21:5437–5447
45. Lopez GN, Turck CW, Schaufele F, Stallcup MR, Kushner PJ 2001 Growth factors signal to steroid receptors through mitogen-activated protein kinase regulation of p160 coactivator activity. *J Biol Chem* 276:22177–22182
46. Feng W, Webb P, Nguyen P, Liu X, Li J, Karin M, Kushner PJ 2001 Potentiation of estrogen receptor activation function 1 (AF-1) by Src/JNK through a serine 118-independent pathway. *Mol Endocrinol* 15:32–45
47. Wiepz GJ, Houtman JC, Cha D, Bertics PJ 1997 Growth hormone attenuation of epidermal growth factor-induced mitogenesis. *J Cell Physiol* 173:44–53
48. Zwijsen RM, Wientjens E, Klompmaker R, van der Sman J, Bernards R, Michalides RJ 1997 CDK-independent activation of estrogen receptor by cyclin D1. *Cell* 88:405–415
49. Zwijsen RM, Buckle RS, Hijmans EM, Loomans CJ, Bernards R 1998 Ligand-independent recruitment of steroid receptor coactivators to estrogen receptor by cyclin D1. *Genes Dev* 12:3488–3498
50. McMahon C, Suthiphongchai T, DiRenzo J, Ewen ME 1999 P/CAF associates with cyclin D1 and potentiates its activation of the estrogen receptor. *Proc Natl Acad Sci USA* 96:5382–5387

51. Hewitt SC, Bocchinfuso WP, Zhai J, Harrell C, Koonce L, Clark J, Myers P, Korach KS 2002 Lack of ductal development in the absence of functional estrogen receptor α delays mammary tumor formation induced by transgenic expression of ErbB2/neu. *Cancer Res* 62: 2798-2805
52. Lin SY, Makino K, Xia W, Matin A, Wen Y, Kwong KY, Bourguignon L, Hung MC 2001 Nuclear localization of EGF receptor and its potential new role as a transcription factor. *Nat Cell Biol* 3:802-808
53. Cordero JB, Cozzolino M, Lu Y, Vidal M, Slatopolsky E, Stahl PD, Barbieri MA, Dusso A 2002 A 1,25-dihydroxyvitamin D down-regulates cell membrane growth and nuclear growth-promoting signals by the epidermal growth factor receptor. *J Biol Chem* 277:38965-38971
54. Klotz DM, Hewitt SC, Ciana P, Raviscioni M, Lindzey JK, Foley J, Maggi A, DiAugustine RP, Korach KS 2002 Requirement of estrogen receptor- α in insulin-like growth factor-1 (IGF-1)-induced uterine responses and *in vivo* evidence for IGF-1/estrogen receptor cross talk. *J Biol Chem* 277:8531-8537
55. Varma H, Conrad SE 2002 Antiestrogen ICI 182,780 decreases proliferation of insulin-like growth factor I (IGF-I)-treated MCF-7 cells without inhibiting IGF-I signaling. *Cancer Res* 62:3985-3991
56. Oesterreich S, Zhang P, Guler RL, Sun X, Curran EM, Welshons WV, Osborne CK, Lee AV 2001 Re-expression of estrogen receptor α in estrogen receptor α -negative MCF-7 cells restores both estrogen and insulin-like growth factor-mediated signaling and growth. *Cancer Res* 61:5771-5777
57. Chan TW, Pollak M, Huynh H 2001 Inhibition of insulin-like growth factor signaling pathways in mammary gland by pure antiestrogen ICI 182,780. *Clin Cancer Res* 7:2545-2554
58. Lee AV, Jackson JG, Gooch JL, Hilsenbeck SG, Coronado-Heinsohn E, Osborne CK, Yee D 1999 Enhancement of insulin-like growth factor signaling in human breast cancer: estrogen regulation of insulin receptor substrate-1 expression *in vitro* and *in vivo*. *Mol Endocrinol* 13:787-796
59. Kahlert S, Nuedling S, van Eickels M, Vetter H, Meyer R, Grohe C 2000 Estrogen receptor α rapidly activates the IGF-1 receptor pathway. *J Biol Chem* 275:18447-18453
60. Dupont J, Karas M, LeRoith D 2000 The potentiation of estrogen on insulin-like growth factor I action in MCF-7 human breast cancer cells includes cell cycle components. *J Biol Chem* 275:35893-35901
61. Lai A, Sarcevic B, Prall OW, Sutherland RL 2001 Insulin/insulin-like growth factor-I and estrogen cooperate to stimulate cyclin E-Cdk2 activation and cell cycle progression in MCF-7 breast cancer cells through differential regulation of cyclin E and p21(WAF1/Cip1). *J Biol Chem* 276:25823-25833
62. Sato T, Wang G, Hardy MP, Kurita T, Cunha GR, Cooke PS 2002 Role of systemic and local IGF-I in the effects of estrogen on growth and epithelial proliferation of mouse uterus. *Endocrinology* 143:2673-2679
63. Tesarik J, Mendoza C 1995 Nongenomic effects of 17 β -estradiol on maturing human oocytes: relationship to oocyte developmental potential. *J Clin Endocrinol Metab* 80:1438-1443
64. Kelly MJ, Wagner EI 1999 Estrogen modulation of G-protein coupled receptors. *Trends Endocrinol Metab* 10:369-374
65. Aronica SM, Kraus WL, Katzenellenbogen BS 1994 Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. *Proc Natl Acad Sci USA* 91:8517-8522
66. Le Mellay V, Grosse B, Lieberherr M 1997 Phospholipase C β and membrane action of calcitriol and estradiol. *J Biol Chem* 272:11902-11907
67. Lieberherr M, Grosse B, Kachkache M, Balsan S 1993 Cell signaling and estrogens in female rat osteoblasts: a possible involvement of unconventional non-nuclear receptors. *J Bone Miner Res* 8:1365-1376
68. Kelly MJ, Levin ER 2001 Rapid actions of plasma membrane estrogen receptors. *Trends Endocrinol Metab* 12: 152-156
69. Pietras R, Szego CM 1977 Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. *Nature* 265:69-72
70. Norfleet AM, Thomas ML, Gametchu B, Watson CS 1999 Estrogen receptor- α detected on the plasma membrane of aldehyde-fixed GH3/B6/F10 rat pituitary tumor cells by enzyme-linked immunocytochemistry. *Endocrinology* 140:3805-3814
71. Razandi M, Pedram A, Greene GL, Levin ER 1999 Cell membrane and nuclear estrogen receptors derive from a single transcript: studies of ER α and ER β expressed in CHO cells. *Mol Endocrinol* 13:307-319
72. Wyckoff MH, Chambliss KL, Mineo C, Yuhanna IS, Mendelsohn ME, Mumby SM, Shaul PW 2001 Plasma membrane estrogen receptors are coupled to endothelial nitric-oxide synthase through G α (i). *J Biol Chem* 276:27071-27076
73. Daub H, Weiss FU, Wallasch C, Ullrich A 1996 Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature* 379:557-560
74. Filardo EJ, Quinn JA, Bland KI, Frackelton Jr AR 2000 Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, gpr30, and occurs via transactivation of the epidermal growth factor receptor through release of HB-EGF. *Mol Endocrinol* 14:1649-1660
75. Filardo EJ, Quinn JA, Frackelton Jr AR, Bland KI 2002 Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. *Mol Endocrinol* 16: 70-84
76. Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, Ullrich A 1999 EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* 402:884-888
77. Carmeci C, Thompson DA, Ring HZ, Francke U, Weigel RJ 1997 Identification of a gene (GPR30) with homology to the G-protein-coupled receptor superfamily associated with estrogen receptor expression in breast cancer. *Genomics* 45:607-617
78. Razandi M, Pedram A, Parks S, Levin ER 2003 Proximal events in signaling by plasma membrane estrogen receptors. *J Biol Chem* 278:2701-2712 (First published 5 November 2002; 10.1074/jbc.M205692200)
79. Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, Lombardi M, Barone MV, Ametrano D, Zanini MS, Abbondanza C, Auricchio F 2000 Steroid-induced androgen receptor-oestradiol receptor β -Src complex triggers prostate cancer cell proliferation. *EMBO J* 19:5406-5417
80. Russell KS, Haynes MP, Sinha D, Clerisme E, Bender JR 2000 Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. *Proc Natl Acad Sci USA* 97: 5930-5935
81. Zhu Y, Bian Z, Lu P, Karas RH, Bao L, Cox D, Hodgins J, Shaul PW, Thoren P, Smithies O, Gustafsson JA, Mendelsohn ME 2002 Abnormal vascular function and hypertension in mice deficient in estrogen receptor β . *Science* 295:505-508
82. Ahola TM, Manninen T, Alkio N, Ylikomi T 2002 G protein-coupled receptor 30 is critical for a progestin-induced growth inhibition in MCF-7 breast cancer cells. *Endocrinology* 143:3376-3384

83. Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK 2000 Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* 407:538–541
84. Yamabhai M, Anderson RG 2002 Second cysteine-rich region of epidermal growth factor receptor contains targeting information for caveolae/rafts. *J Biol Chem* 277:24843–24846
85. Okamoto T, Schlegel A, Scherer PE, Lisanti MP 1998 Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. *J Biol Chem* 273:5419–5422
86. Wong, C-W, McNally C, Nickbarg E, Komm BS, Cheskis BJ 2002 Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with Src/Erk phosphorylation cascade. *Proc Natl Acad Sci USA* 99:14783–14788
87. Lee H, Volonte D, Galbiati F, Iyengar P, Lublin DM, Bregman DB, Wilson MT, Campos-Gonzalez R, Bouzahzah B, Pestell RG, Scherer PE, Lisanti MP 2000 Constitutive and growth factor-regulated phosphorylation of caveolin-1 occurs at the same site (Tyr-14) *in vivo*: identification of a c-Src/Cav-1/Grb7 signaling cassette. *Mol Endocrinol* 14:1750–1775
88. Park WY, Cho KA, Park JS, Kim DI, Park SC 2001 Attenuation of EGF signaling in senescent cells by caveolin. *Ann NY Acad Sci* 928:79–84
89. Song RX, McPherson RA, Adam L, Bao Y, Shupnik M, Kumar R, Santen RJ 2002 Linkage of rapid estrogen action to MAPK activation by ER α -Shc association and Shc pathway activation. *Mol Endocrinol* 16:116–127
90. Razandi M, Pedram A, Levin ER 2000 Plasma membrane estrogen receptors signal to anti-apoptosis in breast cancer. *Mol Endocrinol* 14:1434–1447
91. Kumar R, Wang E-A, Mazumdar A, Talukder AH, Mandal M, Yang Z, Bagheri-Yarmand R, Sahin A, Hortobagyi G, Adam L, Barnes CJ, Vadlamudi RK 2002 A naturally occurring MTA1 variant sequesters oestrogen receptor- α in the cytoplasm. *Nature* 418:654–657
92. Singer CA, Figueroa-Masot XA, Batchelor RH, Dorsa DM 1999 The mitogen activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons. *J Neurosci* 19:2455–2463
93. Watters JJ, Campbell JS, Cunningham MJ, Krebs EG, Dorsa DM 1997 Rapid membrane effects of steroids in neuroblastoma cells: effects of estrogen on mitogen activated protein kinase signalling cascade and c-fos immediate early gene transcription. *Endocrinology* 138:4030–4033
94. Watters JJ, Chun TY, Kim YN, Bertics PJ, Gorski J 2000 Estrogen modulation of prolactin gene expression requires an intact mitogen-activated protein kinase signal transduction pathway in cultured rat pituitary cells. *Mol Endocrinol* 14:1872–1881
95. Kousteni S, Chen JR, Bellido T, Han L, Ali AA, O'Brien CA, Plotkin L, Fu Q, Mancino AT, Wen Y, Vertino AM, Powers CC, Stewart SA, Ebert R, Parfitt AM, Weinstein RS, Jilka RL, Manolagas SC 2002 Reversal of bone loss in mice by nongenotropic signaling of sex steroids. *Science* 298:843–846
96. Migliaccio A, Di Domenico M, Castoria G, de Falco A, Bontempo P, Nola E, Auricchio F 1996 Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *EMBO J* 15:1292–1300
97. Bjornstrom L, Sjoberg M 2002 Signal transducers and activators of transcription as downstream targets of nongenomic estrogen receptor actions. *Mol Endocrinol* 16:2202–2214
98. Wilson MA, Chrysogelos SA 2002 Identification and characterization of a negative regulatory element within the epidermal growth factor receptor gene first intron in hormone-dependent breast cancer cells. *J Cell Biochem* 85:601–614
99. deFazio A, Chiew YE, Sini RL, Janes PW, Sutherland RL 2000 Expression of c-erbB receptors, heregulin and oestrogen receptor in human breast cell lines. *Int J Cancer* 87:487–498
100. Pedram A, Razandi M, Aitkenhead M, Hughes CCW, Levin ER 2002 Integration of the non-genomic and genomic actions of estrogen. Membrane-initiated signaling by steroid to transcription and cell biology. *J Biol Chem* 277:50768–50775 (First published 7 October 2002; 10.1074/jbc.M210106200)



Identification of a Structural Determinant Necessary for the Localization and Function of Estrogen Receptor α at the Plasma Membrane

Mahnaz Razandi,^{1,2} Gordon Alton,³ Ali Pedram,^{1,2} Sanjiv Ghonshani,^{1,2}
Paul Webb,⁴ and Ellis R. Levin^{1,2,5*}

*Division of Endocrinology, Veterans Affairs Medical Center, Long Beach, Long Beach, California 90822¹;
Departments of Medicine² and Pharmacology,³ University of California, Irvine, Irvine, California 92717;
Signal Pharmaceuticals Inc., San Diego, California 92121³; and Diabetes and Metabolic Research
Unit at the University of California, San Francisco, San Francisco, California 94143⁴*

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Estrogen receptors (ER) have been localized to the cell plasma membrane (PM), where signal transduction mediates some estradiol (E2) actions. However, the precise structural features of ER that result in membrane localization have not been determined. We obtained a partial tryptic peptide/mass spectrometry analysis of membrane mouse ER α protein. Based on this, we substituted alanine for the determined serine at amino acid 522 within the E domain of wild-type (wt) ER α . Upon transfection in CHO cells, the S522A mutant ER α resulted in a 62% decrease in membrane receptor number and reduced colocalization with caveolin 1 relative to those with expression of wt ER α . E2 was significantly less effective in stimulating multiple rapid signals from the membranes of CHO cells expressing ER α S522A than from those of CHO cells expressing wt ER α . In contrast, nuclear receptor expression and transcriptional function were very similar. The S522A mutant was also 60% less effective than wt ER α in binding caveolin 1, which facilitates ER transport to the PM. All functions of ER α mutants with other S-to-A substitutions were comparable to those of wt ER, and deletion of the A/B or C domain had little consequence for membrane localization or function. Transfection of ER α S522A into breast cancer cells that express native ER downregulated E2 binding at the membrane, signaling to ERK, and G_i/S cell cycle events and progression. However, there was no effect on the E2 transactivation of an ERE-luciferase reporter. In summary, serine 522 is necessary for the efficient translocation and function of ER α at the PM. The S522A mutant also serves as a dominant-negative construct, identifying important functions of E2 that originate from activating PM ER.

Steroid action is attributed primarily to the regulation of target genes through nuclear receptor binding and transactivation, subsequently producing cell biological effects (42). However, it has increasingly been appreciated that steroids, such as estradiol (E2), act rapidly through nongenomic mechanisms of signal transduction (4, 16, 41). These signaling mechanisms have important consequences for the effects of steroids on cell biology (14, 40). For E2, these effects can occur after the sex steroid binds to plasma membrane (PM) estrogen receptors (ER) (17, 30), which has been demonstrated by immunohistochemistry (36) and by immunoblotting of isolated PM domains (6). Some signaling effects of E2-ER can result from complex interactions with PM growth factor tyrosine kinase receptors, such as the epidermal growth factor receptor (EGFR) (10).

Although the exact sequence of this receptor has not been reported, the membrane ER appears to be very similar, and perhaps identical, to the nuclear receptor. This is based upon the identification of similarly sized nuclear and membrane ER proteins that result from the expression of a single cDNA (and resulting single mRNA) in CHO cells (32). Also, membrane

ER have been localized on vascular smooth muscle, pituitary, and endothelial cells that express endogenous receptors, by using antibodies raised against multiple epitopes of the nuclear ER α (25, 26, 36). However, many questions remain concerning this relatively small population of ER at the cell surface.

The membrane ER has been reported to be G protein linked (32, 50), and E2 binding can activate many signal transduction pathways that emanate from G protein activation. These include kinase and endothelial nitric oxide synthase activation, cyclic AMP (cAMP) and inositol phosphate (IP) generation, and phospholipase C (PLC) stimulation (4, 16, 18, 24, 50). Linkage to G proteins may be direct, as shown in transfected CHO cells expressing ER α or ER β (32) or in endothelial cells (50), but it has also been reported that E2 activates an orphan G protein-coupled receptor (10). Furthermore, it is not clear whether this receptor spans the cell membrane or is predominantly localized within or associated with the membrane bilayer. Membrane ER have recently been shown to exist in discrete caveolar domains of the PM (6, 13). It has recently been found that membrane ER α can physically associate with the caveolar structural coat proteins caveolin 1 and caveolin 2 (31). Caveolin proteins serve as scaffolds, bringing together various signaling molecules within a discrete area of the PM to regulate cytokine-induced signal transduction (3, 27). These include G proteins, nonreceptor and receptor tyrosine kinases (Src, EGFR), and threonine-serine kinases, such as phospho-

* Corresponding author. Mailing address: Medical Service (111-I), Long Beach VA Medical Center, 5901 E. 7th St., Long Beach, CA 90822. Phone: (562) 826-5748. Fax: (562) 826-5515. E-mail: ellis.levin@med.va.gov.

tidylinositol 3-kinase (PI 3-kinase) and Raf. Organization of signaling molecules within a confined space potentially allows E2-ER to modulate a variety of signaling cascades in target cells.

Signal transduction via the membrane ER has increasingly been found to be important for the cell biological effects of this steroid, including the survival and/or growth of breast cancer, bone, and neural cells (5, 7, 14, 24, 34, 49). This receptor has also been implicated in prevention of the inflammatory response to muscle ischemia-reperfusion injury (40), maintenance of the endothelial cell cytoskeleton, and upregulation of vascular cell migration and angiogenesis (33). E2 stimulation of transcription can also be signal dependent, as stimulation of the mitogen-activated protein (MAP) kinase ERK (extracellular regulated kinase) has been shown to be important for transactivation of the *c-fos* and prolactin genes (9, 45, 46). Transcription in response to E2 generation of cAMP has also been reported (4). The precise structural features of ER that facilitate the translocation of this steroid binding protein to the membrane are not known, but such information is important for understanding of the details of estrogen action at the cell surface.

The studies reported here result from our attempt to understand the localization and function of this protein at the PM. To begin this, we partially determined the amino acid structure of the mouse membrane ER α , isolated from CHO cells transfected to express this protein. We identified a serine residue at 522 that is necessary for the optimal localization and function of the sex steroid receptor at the cell surface. In contrast, mutation of this serine had no effect on nuclear ER number, affinity for E2, or E2-induced transactivation function. We also report that expression of the S522A mutant ER α resulted in a dominant-negative action only at the membrane, in cells expressing wild-type (wt) ER α . This mutant abolished several important effects of E2 in breast cancer and can be used as a reagent to deduce the cellular actions of E2 originating from membrane ER α .

MATERIALS AND METHODS

Isolation and partial sequencing of a membrane ER. CHO-K1 cells were transiently transfected with a cDNA for the mouse ER α , as previously described (32). This resulted in the expression of both nuclear and membrane receptors. Twenty plates of ER α -transfected CHO cells were scraped and pelleted at 1,000 $\times$ g, and pellets were resuspended in 20 mM Tris with 1 mM EDTA, 1 mM dithiothreitol, and protease inhibitors. Cells were then centrifuged at 4°C and 8,000 $\times$ g for collection of nuclear receptors, and the supernatant was then ultracentrifuged at 4°C and 100,000 $\times$ g for 1 h. The pellet (membranes) was washed and ultracentrifuged again, and membranes were then further separated by sucrose gradient overlay; fractions 3 to 5 contained the buoyant membranes (with caveolae and rafts) that were pooled for all experiments (31). Briefly, membrane samples were first placed in a tube with an equal volume of a solution containing 85% (wt/vol) sucrose, 25 mM A-morpholine-ethanesulfonic acid, and 0.15 M NaCl and were then overlaid with 8.5 ml of 35% sucrose, topped up with 16% sucrose, and centrifuged at 35,000 rpm (105,000 $\times$ g) for 18 h at 4°C. Ten fractions (1 ml each) were obtained and either further processed or separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by membrane transfer for immunoblotting. The membrane receptors were solubilized in binding buffer (Pierce) containing 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS; Sigma). The purity of the membranes was confirmed by positive immunoblotting for 5'-nucleotidase and caveolin 1 (membrane proteins) and by the lack of detection of transportin and NTF-2 (nuclear proteins) or β -coatomer protein (endosomal/Golgi protein) (38). This was followed by affinity column purification. Briefly, protein G bound to an antibody against ER α (H222) (11) was cross-linked with disuccinimidyl suberate

to make the column. The ER α -containing membrane or nuclear protein was eluted by using proprietary buffers and a proprietary protocol (Pierce). The eluted receptor proteins were dialyzed or concentrated and then analyzed by SDS-PAGE after being run on a 7.5% gel followed by staining. The gel protein bands corresponding to 67 kDa were cut out, trypsin was extracted from the gel, and the bands were then subjected to peptide degradation-mass spectrometry, as previously described (2, 12). This generated peptide sequences from the membrane and nuclear proteins, and these were compared to the known sequences of the classical mouse nuclear ER α .

Site directed mutagenesis and targeting of mouse ER α . We carried out tryptic peptide matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry "sequencing," as described above. At present this has yielded membrane peptide sequences that identically overlap with 20% of the known nuclear receptor and with 20% of our expressed nuclear receptor, supporting the idea that the two receptors are the same (G. Alton, M. Razandi, A. Pedram, and E. Levin, unpublished data). We identified an overlap sequence from amino acid 508 to amino acid 524 that includes a serine at 522. With surrounding residues, this was identified by computer analysis as a potential (although not a classic) palmitoylation site (HMSN). This sequence is present as amino acids 517 to 520 of the human receptor as well. We then mutated the serine at 522 to alanine in mouse pcDNA3-ER α by PCR using the forward primer 5'-CGGCACATGGCTAACA AAGG-3'. As specificity controls, we also mutated the identified serine residues 10 and 582 to alanine by using the forward primers 5'-CCCTTCACACCAAA GCCCGGGGAATGGCCTTGCTGC-3' and 5'-GCTCCACTTCAGCACATG CCTTACAAACCTACTAC-3', respectively. All mutations were confirmed by sequencing at the University of California—Irvine sequencing facility. We additionally subcloned the receptors into a green fluorescent protein (GFP) vector, pEGFPc2 (Clontech, Palo Alto, Calif.), and a multicopy histidine-expressing vector to monitor transfection efficiencies. wt and mutant receptor expression plasmids were then used in studies. To generate nuclear and membrane wt ER α constructs, pcDNA3-mouse ER α was used as a template. Twenty-five cycles of PCR (annealing temperature, 55°C) were performed by utilizing the forward primer 5'-GCCGCTAGCACCATGACCATGACCCCTTCAC3' and the lower primer 5'-GCCACCGGTCTGATCGTGTGGGGAAGCC3'. The PCR product was ligated into pCR2.1 by using the TA cloning kit (Invitrogen, Carlsbad, Calif.) and digested with *Age*I and *Nhe*I. This fragment was subcloned into *Age*I and *Nhe*I sites on the pECFP-Nuc and pECFP-mem vectors (Clontech), yielding ER constructs that were predominantly targeted to either the membrane or the nucleus (confirmed by binding and functional studies).

Receptor binding and cell localization studies. wt and mutant ER α were expressed in CHO cells, and nuclear and membrane fractions were isolated as detailed above and were used for competitive binding assays or signal transduction studies, as previously described (31, 32). Binding studies were repeated at least three times, and the data were used for Scatchard analysis with the LIGAND computer program. Results were combined for statistical comparison by analysis of variance plus Scheffé's test. Additional ER α mutants (HE11G, with the A/B domain deleted; HE19G, with the C domain deleted; and HEG0-537, with helix 12 and the F domain deleted) were provided by Paul Webb and expressed in CHO cells.

For cell localization of wt or S522A mutant ER α , we transiently expressed GFP-tagged fusion proteins for each receptor in CHO cells. CHO cells were grown and transfected on coverslips, and localization of the receptors was examined by laser-scanning confocal microscopy. We also colocalized the receptors at the membrane with endogenous caveolin 1 by using an antibody to this protein (Zymed Laboratories, South San Francisco, Calif.). Each section was processed for GFP-ER α (green), caveolin 1 (second antibody conjugated to Texas red), and colocalized caveolin 1 and ER α (yellow).

Signaling studies. Adenylate cyclase activity in the membrane was determined by measuring cAMP generation, by methods described previously (32), in CHO-K1 cells expressing wt or S522A mutant ER α after the cells had been incubated for 5 min with 10 nM E2. IP generation and ERK (MAP kinase) activation in the CHO cells were also determined as described in detail elsewhere (32). Activation by E2 of an ERE-luciferase reporter in ER-transfected CHO or MCF-7 cells was assessed at 8 h of exposure to 10 nM E2, as previously published (32). Membranes were isolated by sucrose gradient centrifugation (31).

Myristylation, palmitoylation, and PI-PLC studies in CHO-K1 cells. Cells were grown on 100-mm-diameter petri dishes in Dulbecco's modified Eagle medium (DMEM)-F12 medium without phenol red. Twenty-four hours after transfection with ER α , the cells were synchronized overnight and then labeled with [^{3} H]palmitic acid (0.5 μ Ci/ml) or [^{3} H]myristic acid (0.2 μ Ci/ml) for 2 h. The cells were incubated for 8 h in the presence or absence of 10 nM 17 β -E2, washed with cold phosphate-buffered saline, and then lysed in buffer A (50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 100 mM NaCl, 50 mM NaF, 100 μ M phenylmethylsul-

fonyl fluoride, protease inhibitor cocktail, and 0.2% Triton X-100). Nuclear pellets were collected by low-speed centrifugation. Supernatants were centrifuged at $100,000 \times g$ for 30 min to pellet cell membranes. Both pellets were washed twice, once with buffer A and once without detergent. Membranes were further purified by sucrose gradient centrifugation. Membrane and nuclear fractions were denatured in SDS loading buffer followed by gel electrophoresis, fluorography, and autoradiography. For phosphoinositide (PI)-PLC studies, the cells were incubated with 1 U of PI-PLC (Sigma)/ml for 1 h. Cells were washed and lysed, and the membrane and nuclear fractions were collected. Specific, total binding studies were then carried out on 50 μ l of nuclear or membrane protein, incubated in DMEM-F12 medium (with no phenol red), bacitracin (1 mg/ml), and 0.5% bovine serum albumin, and with ^3H -labeled E2 and unlabeled E2 (10^{-11} to 10^{-7} M).

Cyclin D1 protein expression, thymidine incorporation, and kinase activity. MCF-7 cells were transfected with pcDNA3 (control) or ER α S522A, recovered, then synchronized by serum deprivation for 24 h, and then incubated in the presence or absence of 10 nM E2 for 8 h. In some cells, the soluble MEK inhibitor PD98059 (10 μ M) was added to the incubation mixture 30 min prior to the steroid. The cells were then lysed, precleared, boiled, denatured in SDS reducing buffer, and electrophoretically resolved by PAGE. Western immunoblotting was then carried out using a polyclonal antibody (Santa Cruz). Nuclear thymidine incorporation was carried out in nontransfected or transfected MCF-7 cells after synchronization overnight in serum-free medium. All cells were then incubated for 20 h in the absence or presence of 10 nM ^{125}I -E2. In some conditions, the MEK inhibitor PD 98059 (10 μ M) was added prior to the steroid. After 20 h, 0.5 μ Ci of [^3H]thymidine was added for 4 more h, as previously described (32). Cells were then washed and incubated for 10 min with 10% trichloroacetic acid at 4°C, followed by additional washes. Cells were lysed with 0.2 N NaOH overnight, and lysates were counted in a liquid scintillation β -counter. For cdk4 activity, MCF-7 cells transfected with pcDNA3 (control) or ER α S522A were incubated with E2 for 6 h and then lysed. The cell lysate was added to a protein A-Sepharose-conjugated cdk4 antibody (Santa Cruz) and then added to *in vitro* kinase activity tubes containing GST-pRB as a substrate, as previously described (28). This was followed by SDS-PAGE separation and autoradiography. Samples from each condition were assessed for protein loading equivalence, where cdk4 protein was assayed by immunoblotting. For ERK activity assays, transfected or nontransfected CHO, MCF-7, or ZR-75-1 cells were synchronized for 24 h in serum, phenol red, and growth factor-free medium. The cells were then exposed to E2 (10 nM) for 8 min with or without additional substances, and kinase activity was determined by using myelin basic protein (MBP) for the substrate, as previously described (32, 34). For p38 β activity, the cells were incubated with E2 (10 nM) for 20 min and then lysed, and the lysate was immunoprecipitated with protein A-Sepharose conjugated to an antiserum for p38 β . Immunoprecipitated kinases were then added to the protein ATF-1 for *in vitro* kinase assays as previously described (33). All experiments were repeated two to three times.

Protein and ER association studies. Cytosolic fractions of CHO-wt ER α or CHO-ER α S522A were incubated with protein A-Sepharose for 1 h, and supernatants were transferred to fresh tubes containing protein A-Sepharose conjugated to caveolin 1 or ER α antibodies and were incubated for 4 h at 4°C. Immune complexes were washed, boiled, and then separated by SDS-PAGE. After transfer to nitrocellulose filters, the nonspecific proteins were blocked with blocking solution (Bio-Rad) and incubated first with a primary antibody to ER α or caveolin 1 for 2 h and then with a second antibody (Santa Cruz Biotechnology). Bound immunoglobulin G's (IgGs) were visualized using ECL reagents (Amersham) and autoradiography. Portions of the immunoprecipitated ER α or caveolin 1 were immunoblotted for evidence of equal protein loading and equal expression of total ER with the two constructs. In additional studies, MCF-7 cells were transfected to express a GFP-ER α S522A protein or GFP alone. After overnight recovery, the cells were lysed and immunoprecipitated with an antibody to GFP, followed by immunoblotting with antibodies to Src, Ras, and Raf proteins (Santa Cruz). In CHO cells, His-wt ER α or GFP-ER α S522A was singly or doubly expressed. To detect homo- or heterodimerization in these cells, the lysate was immunoprecipitated with an antibody to His, followed by blotting with an antibody to GFP, or in reverse order. All studies were repeated at least three times.

RESULTS

Comparison of wt and S522A mutant ER α binding after expression in CHO cells. We first isolated the mouse ER α in the PM after expression in CHO cells and partially sequenced

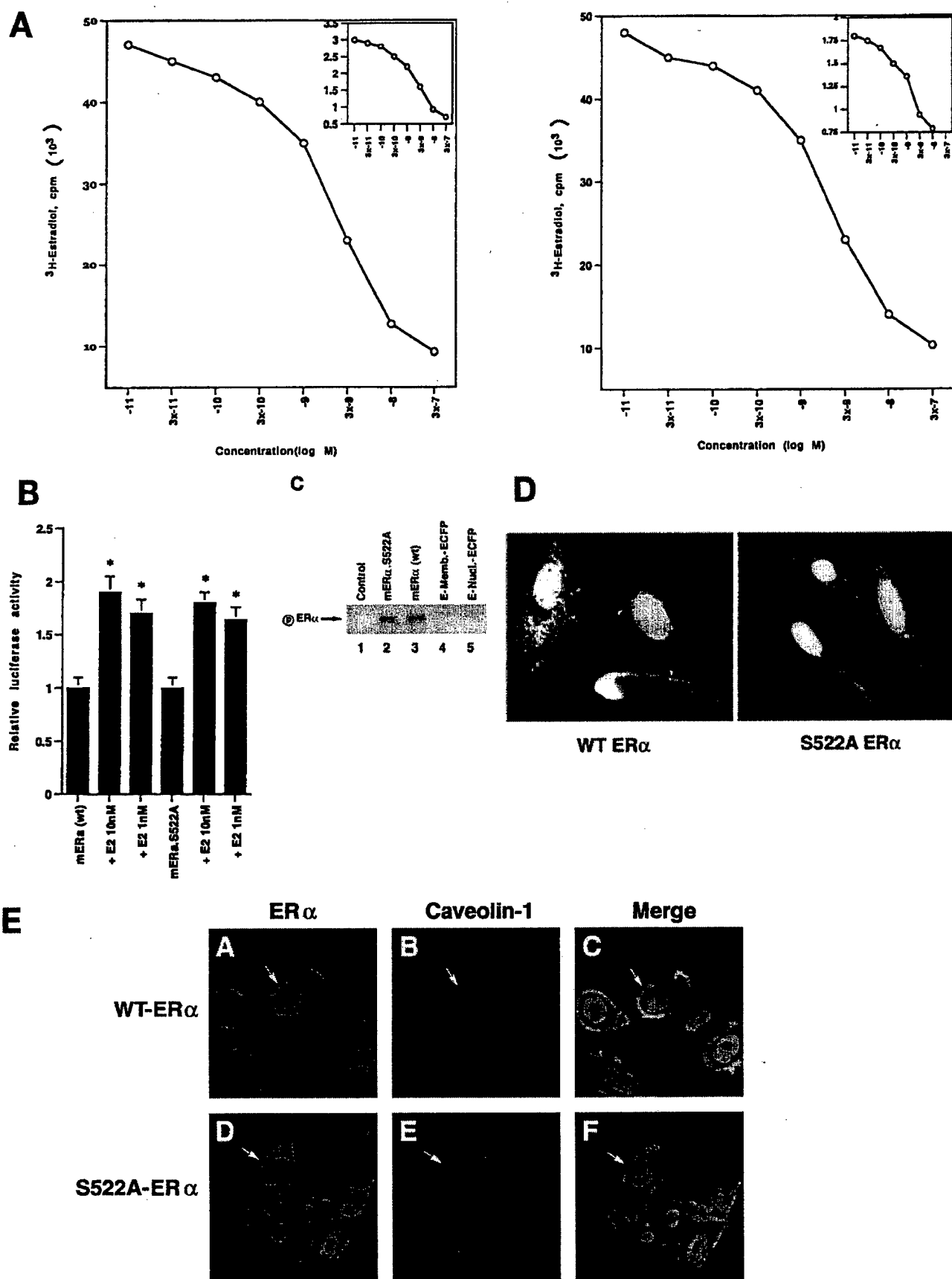
the protein by peptide degradation-MALDI mass spectroscopy (Razandi et al., unpublished). We identified a peptide (LA QLLILSHIRHMSNK) that corresponds to a portion of the C terminus in the known mouse ER α sequence (2), beginning with amino acid 508. Furthermore, HMS (boldfaced in peptide sequence) was noted by computer modeling as a possible, but not classic, palmitoylation site (35). We therefore asked whether the ER was palmitoylated at this site (see below), and we also mutated the critical serine at amino acid position 522 to alanine within the mouse ER α cDNA. Additional S-to-A mutations at residues 10 and 582 were created by site-directed mutagenesis, for comparison to mouse ER α S522A (48) and to support the specificity of any findings.

We then expressed the wt and S522A mutant ER α constructs in CHO cells and carried out competitive binding studies in both nuclear and membrane compartments. By Scatchard analysis of the binding data (Fig. 1A), we found that the receptor affinity for E2 (K_d) and the receptor number (B_{max}) were very similar for the two ER α receptors in the nucleus (Table 1). Similar transfection efficiencies were demonstrated using GFP fusion constructs (data not shown). We also determined whether the function of the mutant nuclear ER α differed from that of the wt. We therefore cotransfected CHO cells with either wt or S522A mutant ER α and an ERE-luciferase reporter (32) and determined the response to E2. We found that the two receptors were comparably capable of responding to E2 with an upregulation of reporter activity (Fig. 1B). These data indicate that the replacement of S with A at residue 522 does not affect the quantity of nuclear receptor localization, its binding affinity for E2, or the transcriptional response to the steroid.

In contrast, binding experiments revealed that expression of the S522A mutant ER α resulted in 62% fewer receptors (B_{max}) in the PM than expression of wt ER α (Fig. 1A insets; Table 1). This was found in three separate binding experiments, where the reduction ranged from 57 to 65%. The binding affinities (K_d) for E2 at the membrane were comparable for wt and mutant receptors. Thus, serine 522 is an important determinant for full membrane localization of ER α .

It is possible that serine 522 is a phosphorylation site, although this would not be a common mechanism for membrane localization. By mass spectroscopy, there was no evidence of phosphorylation on this residue. We also expressed the full-length wt ER, the S522A mutant, or the E domain (ligand binding domain) of wt ER α in CHO cells, targeting the E domain to both nuclear and membrane locations. As seen in Fig. 1C, the entire receptor is phosphorylated at serine/threonine residues, but we find no evidence that the intact E domain is similarly phosphorylated, either when targeted to the membrane or when targeted to the nucleus.

To visualize the receptor at the membrane, we expressed GFP-tagged wt or S522A mutant ER α in CHO cells and detected membrane localization by confocal microscopy. As seen in Fig. 1D, wt receptor expression clearly reveals a population of membrane-localized sex steroid binding proteins, while the mutant receptor does not. Both show a dense nuclear population. We also examined colocalization of the wt or mutant ER α at the membrane with caveolin 1. In Fig. 1E, wt ER α was clearly seen at the membrane (arrow, panel A), in contrast to sparse membrane expression of ER α S522A (panel D). Caveo-



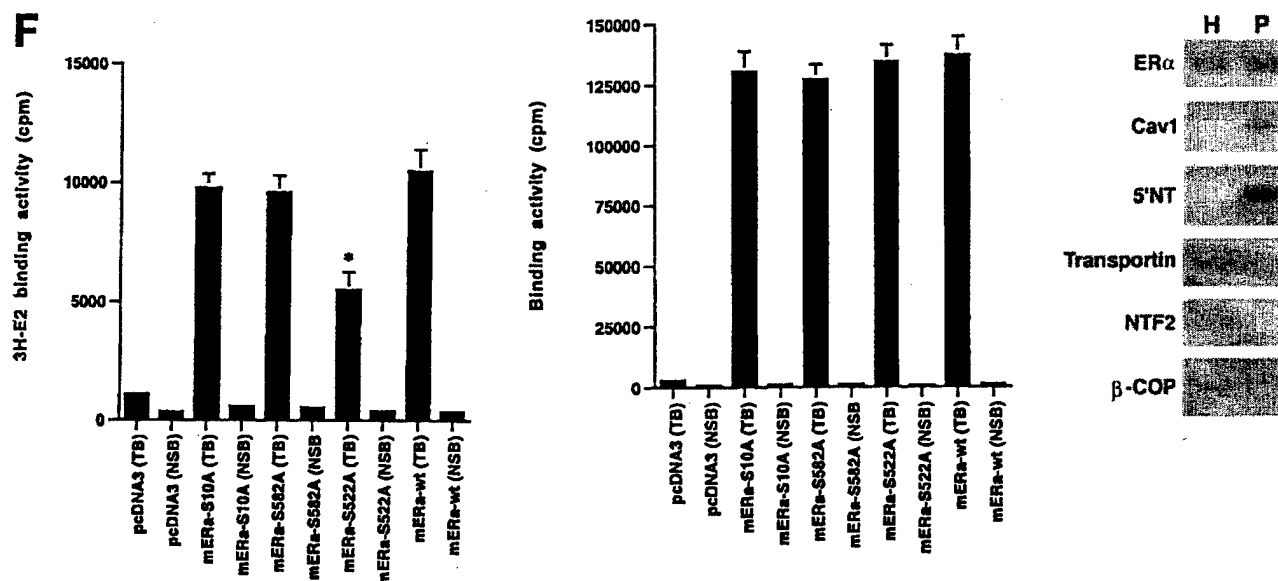


FIG. 1. (A) Competition binding of 17β - ^3H E2 to nuclear wt (left) or S522A mutant (right) ER α transfected into CHO-K1 cells. (Inset) Binding to cell membrane ER α . Data are transformed for Scatchard analysis by using the LIGAND program. Data shown here are from a representative study; results from three separate experiments were combined to create Table 1. (B) Transactivation of an ERE-luciferase reporter construct coexpressed in CHO cells with mouse wt ER α or the S522A mutant. Data were determined at 6 h after incubation with either 1 nM E2, 10 nM E2, or no steroid. *, $P < 0.05$ for the wt or the S522A mutant alone versus the same construct plus E2 (for data combined from three experiments). (C) Serine/threonine residues in the full-length receptor, but not serine 522 in the E domain of ER α , are phosphorylated. Western blotting utilized a specific antibody to serine/threonine residues (Sigma) from lysates of CHO cells transfected to express either the full-length receptor or the E domain targeted to the PM or the nucleus. (D) Membrane localization of GFP-tagged wt ER α or S522A mutant ER α expressed in CHO cells. Arrows indicate a greater membrane localization for wt ER α . Dense nuclear populations for both receptors are seen. (E) Colocalization of wt ER α with caveolin 1 at the membrane, but markedly less colocalization of ER α S522A. Results of a representative study are shown. Arrows indicate differential ER expression (green) at the membrane (panels A and D) and equal caveolin 1 expression (red) (panels B and E). The strong colocalization of wt ER α and caveolin 1 (yellow) (arrow, panel C) is not seen for ER α S522A (arrow, panel F). (F) Total specific binding of labeled E2 to membranes (left) or nuclei (center) in CHO cells expressing either S10A, S582A, or S522A mutant ER α or wt ER α . Data are combined from three experiments. *, $P < 0.05$ for ER α S522A versus wt ER α or other S-to-A mutant receptors. (Right) Protein blot demonstrating the purity of the membrane preparation. Caveolin 1 (Cav1) and 5' nucleotidase (5'NT) are integral membrane proteins, while transportin and NTF-2 are nuclear proteins. β -COP is a Golgi protein.

lin 1 was clearly visualized at the membrane (Fig. 1E, panels B and E). Colocalization of membrane wt ER α with caveolin 1 (Fig. 1E, panel C, arrow) was also seen for the S522A receptor (panel F), but the latter showed decreased amounts colocalized, reflecting a decreased number of receptors at the membrane.

We next compared the binding of wt or S522A mouse ER α to that of S10A and S582A ER α constructs expressed in CHO cells (Fig. 1F). Total specific binding of E2 was determined in the nucleus and PM and revealed that both of the two additional mutant receptors were very similar to the wt receptor in both compartments. By comparison, S522A expression again exhibited significantly lower binding at the membrane only. These data indicate the specificity of S522 for ER localization at the cell surface.

Dissection of the contribution of other domains of ER α to membrane localization and function. It is possible that elements contained within other domains of ER α contribute importantly to membrane localization. In this respect, Schlegel et al. (37) have recently shown that residues 1 to 282 (the A/B and C domains) of human ER α bind to caveolin 1, a largely membrane localized protein that facilitates membrane localization of ER (31) and that, when overexpressed, promotes nuclear ER localization and transcriptional action. We therefore asked

whether mutant ER α that lacked either the A/B or the C domain was capable of localizing to the PM and signaling to ERK. We compared the effects of CHO cells expressing these deletion or truncation mutants to those of CHO cells expressing wt ER α . We found that HE11G (with the A/B domain deleted) and H19G (with the C domain deleted) were comparable to wt ER α , both in specific binding of E2 at the cell membrane and in ERK activation by the steroid (Fig. 2). In contrast, a mutant with helix 12 and the F domain deleted,

TABLE 1. Binding characteristics of wt or S522A mutant mouse ER α expressed in the nuclei and membranes of CHO-K1 cells

Localization	K_d (nM) ^a	B_{max} (pmol/mg of protein) ^a
Nuclear		
wt ER α	0.26 $\pm$ 0.02	422 $\pm$ 38*
Mutant ER α	0.25 $\pm$ 0.02	395 $\pm$ 26*
Plasma membrane		
wt ER α	0.28 $\pm$ 0.04	17.2 $\pm$ 3.8†
Mutant ER α	0.27 $\pm$ 0.03	6.6 $\pm$ 0.26

^a Data are from Scatchard analysis of competitive binding studies and are means $\pm$ standard errors of the means for three separate experiments combined. *, $P < 0.05$ for B_{max} of corresponding nuclear versus membrane ER α . †, $P < 0.05$ for B_{max} of wt ER α versus S522A mutant ER α .

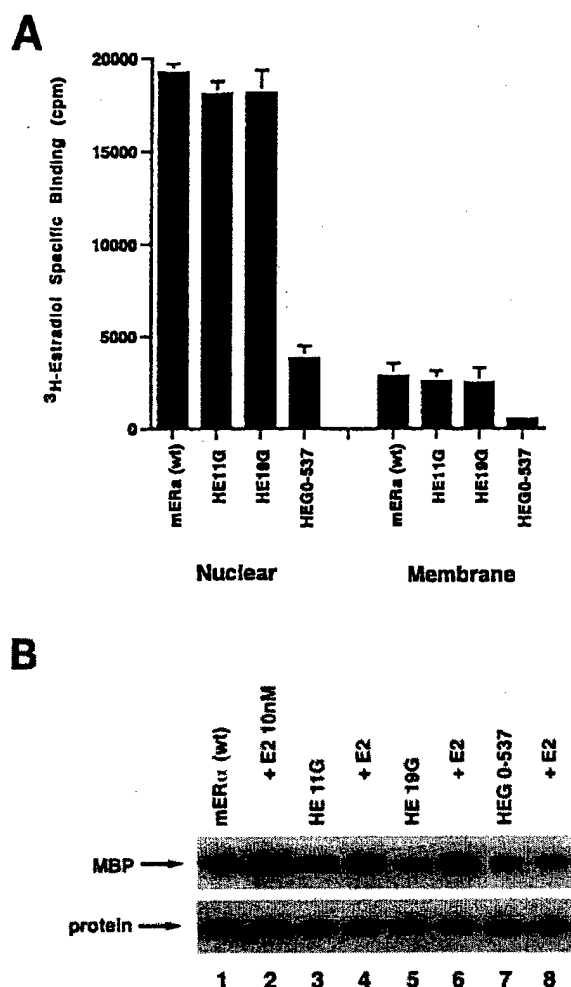


FIG. 2. (A) Binding of E2 to the nuclei and membranes of CHO cells expressing either HE11G (A/B domain deleted), HE19G (C domain deleted), or HEG0-537 (helix 12 and F domain deleted) ER α or wt ER α . The study was repeated. (B) ERK activation in response to E2 in CHO cells expressing either wt ER α or a deletion mutant. Activity was determined after 8 min of incubation with 10 nM E2. MBP was used as a substrate for ERK activity. Total ERK protein is shown on the immunoblot beneath the activity results.

HEG0-537 (truncated at residue 537), specifically bound little E2 in either the nuclear or the membrane compartment and did not support E2 activation of ERK. Thus, the A/B and C domains do not contribute to membrane ER localization and signaling by E2. However, deleting a small but important region within the E domain (in conjunction with loss of the F domain) has a profound effect on E2 binding to any ER pool, as well as on membrane function. Further understanding of the specific residues within the E domain that are required for ER localization at the membrane will require the characterization of a very extensive series of conservative mutations within this region. These results support a focus on the E domain for further understanding of the compartmentalization of ER.

ER α S522A is less capable than wt ER α of signaling from the membrane. It was important to ascertain whether the loss of membrane receptors resulting from expression of the S522A mutant also affected signal transduction. We therefore com-

pared ERK (MAP kinase) activation by E2 in CHO cells expressing wt or mutant ER α . E2 significantly stimulated ERK activity after 8 min of exposure to CHO cells expressing wt ER α (Fig. 3A, left). However, ERK activation in response to E2 was reduced by 68% in CHO cells expressing ER α S522A (relative to activation in cells expressing wt ER α) (Fig. 3A, left; compare lanes 2 and 4). Activation of ERK by E2 was further compared in CHO cells that expressed wt or S10A or S582A mutant receptors. Consistent with the binding data, the additional serine mutants were nearly identical to the wt in activation of this MAP kinase (Fig. 3A, right).

We then examined cAMP generation, reflecting adenylate cyclase activation in the membrane, and found that E2 was 57% less capable of generating this cyclic nucleotide in S522A mutant-expressing than in wt ER α -expressing CHO cells (Fig. 3B). Generation of cAMP often arises from G α s stimulation, which was previously demonstrated in response to membrane ER activation by E2 (32). Finally, we measured IP generation (Fig. 3C) and found a significant (53%) difference in production between cells expressing the two types of ER α . IP generation commonly results from the activation of G α q, which was previously shown to be stimulated by E2 activation of membrane ER expressed in CHO cells (32). These data indicate that the reduction in membrane ER levels seen with S522A protein expression has significant functional consequences, and they further support the idea that E2 activates signal transduction through the membrane (and not the nuclear) receptor. To further support the latter concept, we subcloned the full-length wt ER α into vectors that contain membrane or nuclear localization signals and also express a GFP fusion protein (ECFP; Clontech). We then expressed in CHO cells either nontargeted wt ER α or wt ER α targeted either to the membrane or to the nucleus. As seen in Fig. 3D, expression of the nontargeted wt ER α and especially the membrane-targeted receptor supported rapid ERK activation by E2. In contrast, there was no activation of ERK in CHO cells expressing nucleus-targeted ER. Combined with previous experiments targeting the E domain to the membrane or nucleus (31), these data show that it is the membrane ER that supports rapid kinase activation in response to E2.

Palmitoylation, myristylation, and glucosylphosphoinositol (GPI) anchor studies addressing ER localization at the membrane. A number of posttranslational (or cotranslational) processes have been found to facilitate the movement and anchoring of proteins in the PM. To determine whether any of these alterations helped explain how ER localized to the membrane, we examined lipid modifications of ER. We expressed the wt mouse ER α in CHO cells and then labeled the cells with [^{3} H]palmitate or myristic acid. As expected, there was no uptake or incorporation of either lipid into the nuclear ER α . Furthermore, we found that there was no incorporation of myristate into the membrane ER α , consistent with the lack of a consensus myristylation site determined either from our partial sequence of membrane ER α or from viewing the known full-length sequence (48). Similarly, a possible but nonclassic palmitoylation site was identified from a peptide corresponding to a region in the mouse ER α C terminus, encompassing serine 522. However, in the membrane, there was no specific incorporation of palmitate into ER α in either the presence or the absence of E2 (data not shown). Other modifications, such

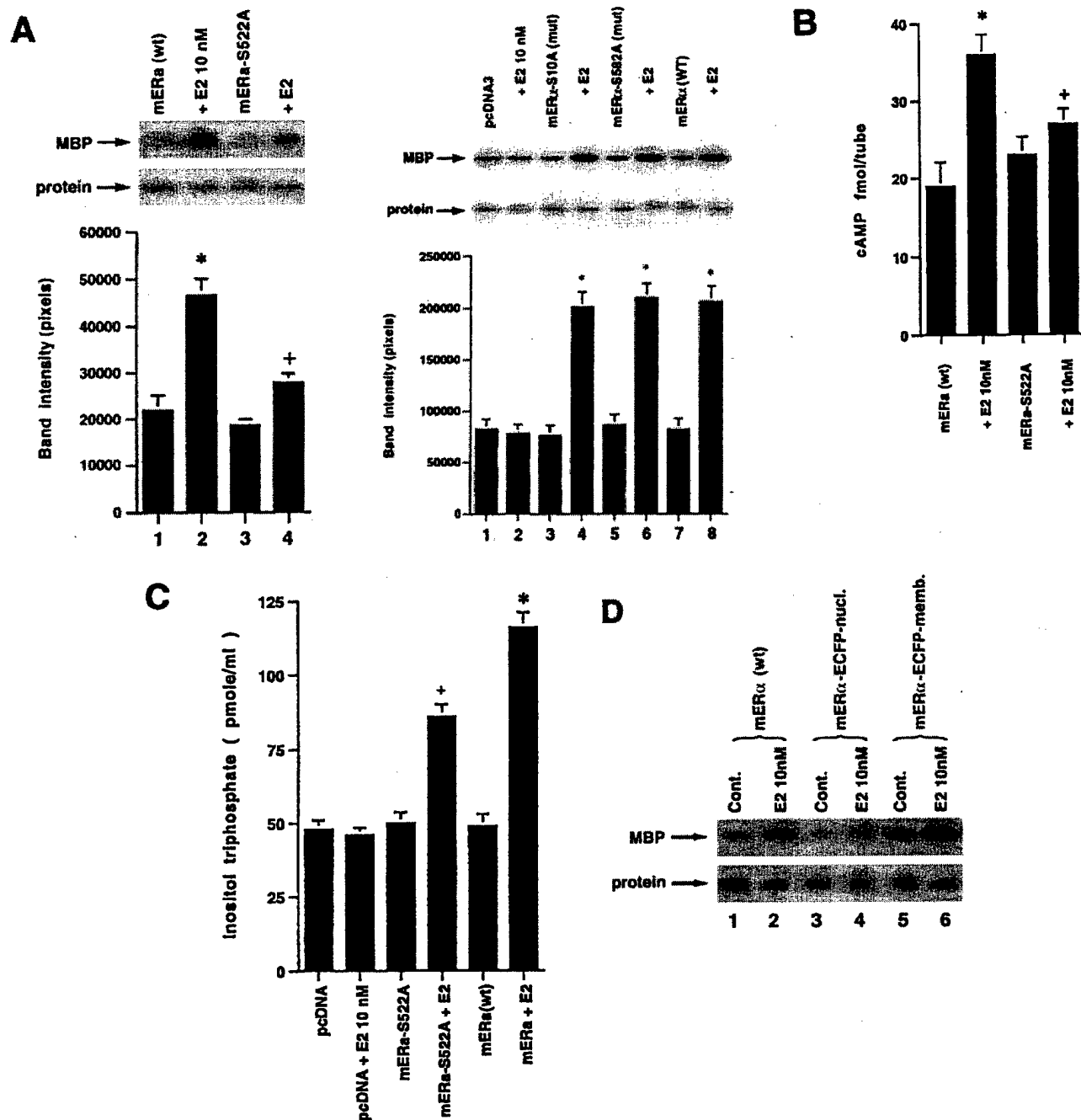


FIG. 3. (A) (Left) ERK activity is stimulated by E2 in CHO cells expressing wt ERα but less so in CHO cells expressing S522A mutant ERα. *, $P < 0.05$ for E2 versus the control (mouse ERα [mERα] without E2) in three combined experiments; +, $P < 0.05$ for ERK response to E2 in CHO cells expressing wt ERα (lane 2) versus ERα S522A (lane 4) in three combined experiments. (Right) Comparable stimulatory effects by E2 on ERK activity in CHO cells expressing wt ERα or the S10A or S582A mutant (lanes 3 to 8). Lanes 1 and 2 show that the intrinsic ERK activity of CHO cells expressing the empty vector, pcDNA3, cannot be stimulated by E2, due to a lack of endogenous ER. *, $P < 0.05$ for control versus E2. (B) Generation of cAMP in response to E2 in CHO cells expressing wt or S522A mutant ER. (C) IP3 generation in response to E2 in the above cells. Bar graph data are means $\pm$ standard errors of the means from triplicate determinations per experiment and are from two (cAMP) or three (IP3) combined studies. (D) Targeting ER to the membrane but not the nucleus allows E2 to rapidly activate ERK. CHO cells were transfected to express either nontargeted wt mERα or wt mERα targeted to the nucleus (mERα-ECFP-nucl.) or the membrane (mERα-ECFP-memb.). Cont., control. The study was repeated.

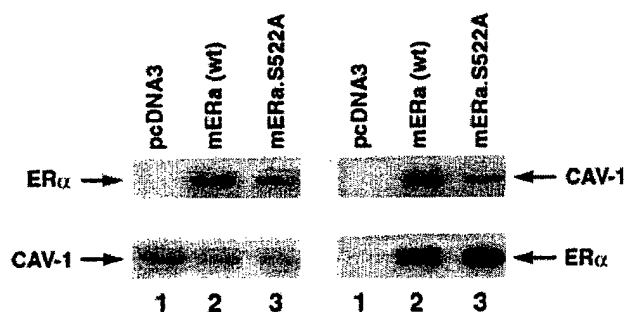


FIG. 4. ER α and caveolin 1 (CAV-1) association in the cytoplasm. CHO-K1 cell cultures (100-mm-diameter dishes) were transfected with 10 μ g of wt or S522A mutant ER α plasmid DNA. The cells were lysed, and immunoprecipitation for caveolin 1 was carried out, followed by immunoblotting for ER α (left panels); or the order was reversed (right panels). Results shown are representative of three experiments. Caveolin 1 and ER α immunoblots are shown (lower panels) to demonstrate equal gel protein loading and equal expression of the two ER. mER α , mouse ER α .

as farnesylation or geranylgeranylation, usually require concomitant palmitoylation and occur at the very end (usually the N terminus) of the protein. No such sites were identified. Thus, we conclude that ER α is probably not posttranslationally lipidated to effect membrane translocation.

Addition of GPI to a protein in the Golgi complex serves to anchor such modified proteins in the extracellular leaflet of the PM (21). The PI-PLC enzyme cleaves GPI-modified proteins and therefore releases these membrane proteins into the culture medium, so that they cannot be detected by binding ligand at the cell surface. We found that treatment of the ER-expressing CHO cells with this phospholipase did not change the binding of E2 to ER in the PM. Furthermore, the anchoring of ER in the outer leaflet of the PM would generally preclude its localization in the caveolae, a membrane domain where ER has now been detected (6, 13). Thus, it is unlikely that ER undergoes this posttranslational (cotranslational) modification.

Interactions of wt or S522A mutant ER α with caveolin 1. It was recently reported that endogenous ER physically associates with the caveolin 1 protein in both the PM and cytosol of endothelial, vascular smooth muscle, and MCF-7 cells (31). Furthermore, expression of full-length caveolin 1 in MCF-7 or Caco-2 cells facilitates the movement of ER from the cytosol to the PM. Thus, ER-caveolin binding is important for the ability of ER to localize to the PM. We therefore examined whether the S522A mutant receptor bound to caveolin 1 comparably to wt ER α . This was accomplished in CHO cells, where we expressed the two ER constructs and utilized the endogenous caveolin in these cells. The ER was immunoprecipitated from the CHO cells, followed by immunoblotting for caveolin 1 (and vice versa), and association was examined in the absence of E2. In the cytosol of CHO cells, expression of either wt or S522A mutant ER α resulted in the receptor complexing with endogenous caveolin 1. However, the association of ER α S522A with caveolin 1 was 60% lower than that of wt ER α (Fig. 4). Importantly, as shown, the total ER levels expressed from the two vectors were comparable. These data are compatible with the idea that S522 is important for binding to caveolin 1, a protein

that facilitates the membrane localization of the steroid binding protein (31). Thus, we have identified a mechanism to explain why ER α S522A is poorly localized to the PM.

Expression of S522A inhibits endogenous membrane ER function. We then asked whether the expression of ER α S522A interferes with the function of endogenous E2-ER signaling from the membrane. To test this hypothesis, we transiently expressed ER α S522A in MCF-7 and ZR-75-1 breast cancer cells. It was previously shown that E2 induced rapid signaling from membrane ER in these cells (33). In MCF-7 cells transfected to express pcDNA3 (control), E2 caused a twofold activation of ERK activity via the endogenous membrane ER (Fig. 5A, left; compare lane 1 with lane 2). In contrast, ER α S522A-expressing cells responded to E2 with 61% less activation of ERK (Fig. 5A, left; compare lanes 1 and 2 with lanes 3 and 4). Comparably, ER α S522A expression resulted in a 70% decrease in ERK activation in E2-treated ZR-75-1 cells (Fig. 5A, right; compare lanes 1 and 2 with lanes 3 and 4). We also determined that activation of ERK by epidermal growth factor (EGF) or IGF-1 in MCF-7 cells (Fig. 5B, first three columns) was not significantly affected by expression of the mutant ER (last three columns). This demonstrates the specific action of ER α S522A to impair only E2-ER signaling, and it also indicates that signaling by the two growth factors does not require an intact membrane ER signaling system. To further establish the specificity of these results, we expressed the S10A mutant in MCF-7 cells. There was no difference in ERK activation in response to E2 between cells expressing only endogenous ER (pcDNA3 transfected) and the same cells additionally transfected with ER α S10A (data not shown).

The inability of ER α S522A to fully localize to the membrane contrasted with the normal amount and function of nuclear ER when this construct was expressed in CHO cells. We therefore determined the specificity of S522A to serve as a dominant-negative protein in MCF-7 cells for membrane but not nuclear ER. MCF-7 cells were transiently transfected with an ERE-luciferase reporter in the presence or absence of co-expression of ER α S522A or pcDNA3. In pcDNA3-expressing cells, E2 caused a dose-related, 2.5-fold maximal stimulation of luciferase function (Fig. 5C). When the S522A mutant was expressed in these cells, transactivation of this reporter by E2 was comparable. This indicates that expression of the mutant ER α did not affect endogenous nuclear ER function.

To understand the cell biological effects of S522A expression and the role of the endogenous membrane ER α , we examined the ability of estrogen to promote cyclin D1 protein expression and cdk4 activation in MCF-7 cells. It has previously been shown that, in response to growth factors, signaling through ERK to Ets protein phosphorylation transactivates the cyclin D1 promoter and stimulates cyclin D1 protein synthesis (1, 15). E2 has been shown to stimulate cyclin D1 transcription and protein synthesis (47). We found that E2 was capable of increasing cyclin D1 protein levels nearly threefold (Fig. 6A). This was significantly related to ERK activation, since the MEK inhibitor PD98059 substantially prevented this effect. It was previously shown that PD98059 completely blocked E2 activation of ERK in MCF-7 cells (34). Importantly, expression of ER α S522A inhibited the E2-induced increase in cyclin D1 protein levels by 68%. Thus, the ability of ER α S522A to inhibit ERK activation arising from the endogenous mem-

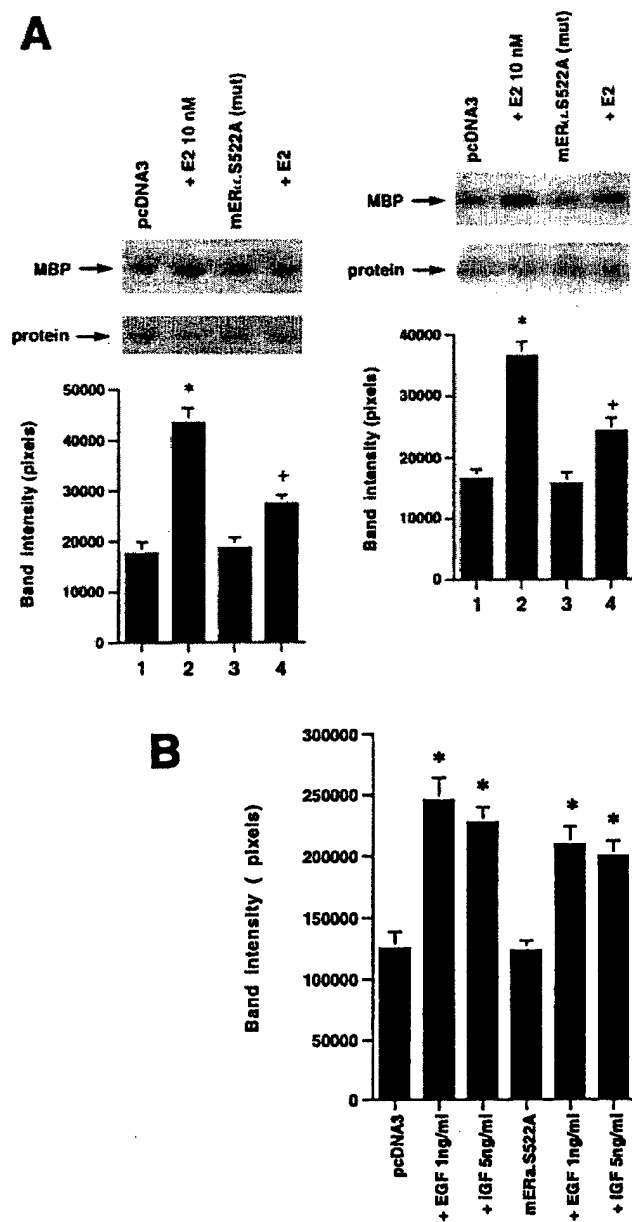


FIG. 5. (A) Expression of ER α S522A inhibits E2 activation of ERK. MCF-7 (left) or ZR-75-1 (right) breast cancer cells (which express endogenous ER) were transfected transiently to express either pcDNA3 (control) or S522A mutant ER α . The cells were then exposed to 10 nM E2 for 8 min, after which they were lysed, and immunoprecipitated ERK was assayed for activity by using MBP as a substrate. Precipitated ERK protein is shown in the lower gels, and the bar graphs each reflect three experiments combined. *, $P < 0.05$ for pcDNA3 in the absence versus the presence of E2; +, $P < 0.05$ for comparison of E2 treatments of pcDNA3-expressing versus ER α S522A-expressing cells. (B) ER α S522A does not impair EGF or IGF-1 activation of ERK. Data from three experiments are combined. *, $P < 0.05$ for pcDNA3-transfected or ER α S522A-expressing MCF-7 cells in the absence versus the presence of EGF or IGF-1. (C) E2 comparably activates an ERE-luciferase reporter in untransfected MCF-7 cells and MCF-7 cells transfected to express ER α S522A. Bar graph shows results for three experiments combined. *, $P < 0.05$ for pcDNA3- or ER α S522A-transfected MCF-7 cells without versus with E2.

brane ER greatly contributed to the inhibition of the increase in cyclin D1 protein levels.

We then determined the effect of E2 signaling on cdk4 activity. We found that E2 stimulated the important phosphorylation of the retinoblastoma (Rb) protein by this kinase 2.5-fold (Fig. 6B). Inactivation of Rb results from its phosphorylation mainly by cyclin D1-cdk4 and possibly by cyclin E-cdk2 and allows G₁/S progression in many cell types (39). Expression of S522A resulted in a 70% decrease in the ability of E2 to activate cdk4 activity. The results indicate that signaling from the membrane ER is important for a G₁ event that is essential to breast cancer cell cycle progression.

We also assessed G₁/S progression, determined by thymidine incorporation into DNA. It has previously been shown in MCF-7 or ER-expressing CHO cells that E2 stimulation of

DNA synthesis is partly regulated through the ERK signaling pathway (5, 32). We found here that E2 caused a 70% increase in thymidine incorporation into DNA, a marker of the S phase. Two-thirds of this increase was blocked by PD98059. Thus, E2 utilizes several mechanisms to stimulate G₁/S progression, but ERK activation is the most important. Upon expression of ER α S522A, E2-induced thymidine incorporation was significantly reduced, by 50%. Thus, expression of the mutant ER α affirms the participation of the membrane steroid receptor in this event.

It would be important to know if the dominant-negative effect of the S522A mutant ER α extended to other cells. It was previously shown that E2 activates p38 β MAP kinase in endothelial cells through endogenous membrane ER and that this leads to the angiogenic and cell survival effects of E2 in these

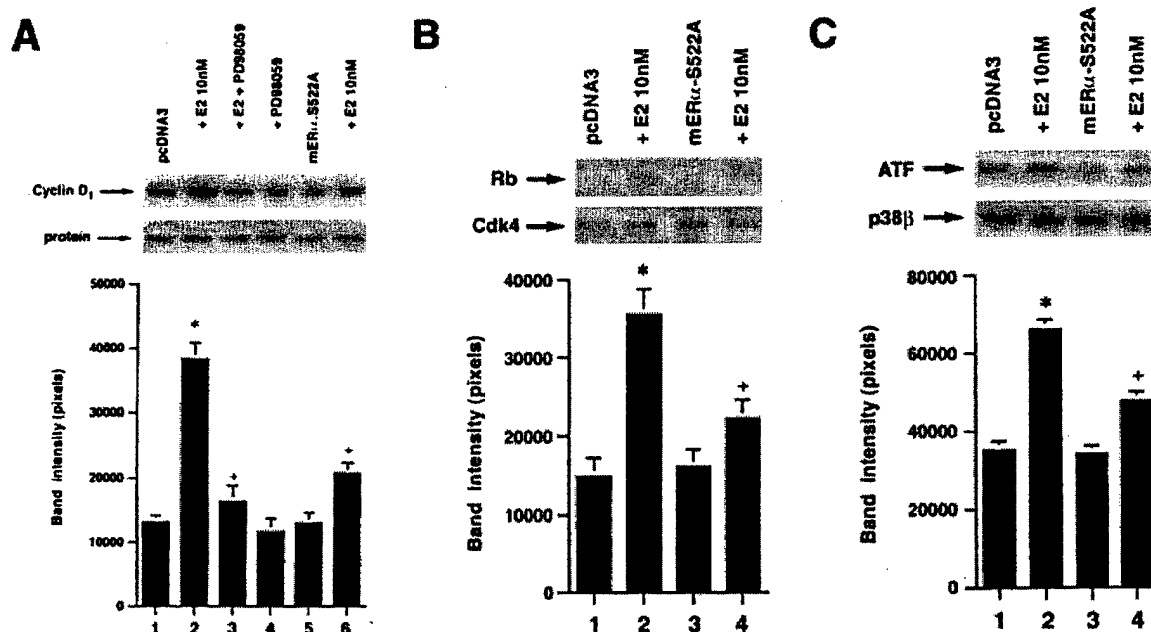


FIG. 6. (A) Cyclin D₁ expression is increased in response to E₂ and is dependent on ERK activation in wt MCF-7 cells. MCF-7 cells transfected to express ERα S522A show a lower response to 100 nM E₂. Data are representative of three experiments, which were combined for the bar graph. *, $P < 0.05$ for pcDNA3-expressing cells without versus with E₂; +, $P < 0.05$ for cells incubated with pcDNA3 plus E₂ versus the same condition plus 10 μ M PD 98059, or versus cells cotransfected with ERα S522A. (B) cdk4 activity is significantly downregulated by ERα S522A in MCF-7 cells. Cells were transfected with pcDNA3 or the mutant ER and exposed to 10 nM E₂ for 6 h. cdk4 kinase was immunoprecipitated, and an in vitro assay of activity was accomplished by using Rb protein as a substrate. Bar graph data are from three experiments combined. (C) E₂-stimulation of p38β activity in endothelial cells is inhibited by ERα S522A. Transfected endothelial cells were incubated with E₂ for 20 min, and p38β activity was determined against the substrate protein ATF-1. Data are from three experiments.

cells (33). Here, we report that E₂ activation of p38β in endothelial cells is 60% reduced when ERα S522A is expressed (Fig. 6C). Thus, this mutant ER may be a useful tool for determining the contributions of the membrane ERα to various cell-signaling and biological functions.

Mechanisms of ERα S522A inhibition of endogenous ER function. How might ERα S522A inhibit endogenous ER function? One possibility to explain the dominant-negative effect of ERα S522A is that it might heterodimerize with wt ER. Dimerization is necessary for ER to transactivate genes (43), and heterodimerization between ERα and ERβ has been reported to inhibit ERα function (29). Since the S522A mutant does not translocate effectively to the PM, it could potentially bind and sequester the endogenous receptor, thus interfering with its signaling function. To assess possible heterodimerization, we expressed GFP-tagged ERα S522A and His-tagged wt ERα in CHO cells and performed pull-down studies. After lysis, the cell extracts were immunoprecipitated and blotted with anti-GFP and anti-His antibodies, in both orders. After E₂ treatment of cells where either or both tagged forms of the receptor were expressed, we found evidence for homodimerization and heterodimerization of wt and mutant ERα (Fig. 7A). As specificity controls, the fourth lanes show a lack of ER when His-tagged wt ERα is expressed and immunoprecipitated but blotting is done with an antibody to GFP (Fig. 7A, left), and when GFP-tagged ERα S522A is expressed but blotting is done with an antibody to His (Fig. 7A, right). Furthermore, S522A mutant ERα was as capable as wt ERα of dimerizing to wt ERα (Fig. 7A, left). These data indicate that ERα S522A can bind to wt

ERα, thereby potentially sequestering or otherwise limiting endogenous receptor signaling from the membrane.

To further examine this mechanism, we determined the membrane localization of wt ERα in CHO cells transfected to express equal amounts of either (i) GFP-tagged wt ERα plus His-tagged wt ERα or (ii) GFP-tagged wt ERα plus His-tagged ERα S522A. As seen in Fig. 7B, expression of ERα S522A substantially decreased the membrane localization of GFP-tagged wt ERα. By contrast, nuclear receptor expression was not different. Thus, ERα S522A inefficiently translocates to the cell surface and also prevents wt ERα PM localization after heterodimerization. These results provide a mechanism for the dominant-negative action of the mutant ERα, but other effects are tenable.

Membrane wt ERα and S522A mutant ERα bind equally to signaling molecules. It is also important to consider that mutation of serine 522 to alanine might disturb the inherent ability of membrane ER to associate with important signaling molecules. This could contribute to the differential signaling from the membrane by wt versus S522A mutant ERα. To investigate this, we transfected CHO cells to express either wt or S522A mutant ERα. We immunoprecipitated the ER from membrane preparations and then normalized the proteins for equivalent amounts of receptor(s), as indicated by Western blotting (Fig. 7C). We then took separate (normalized) aliquots of immunoprecipitated wt ERα or ERα S522A and immunoblotted the aliquots for Ras or Raf. We found that the two receptors associated equally with the Ras or Raf signaling molecules in the presence of the steroid (Fig. 7C, left). Control

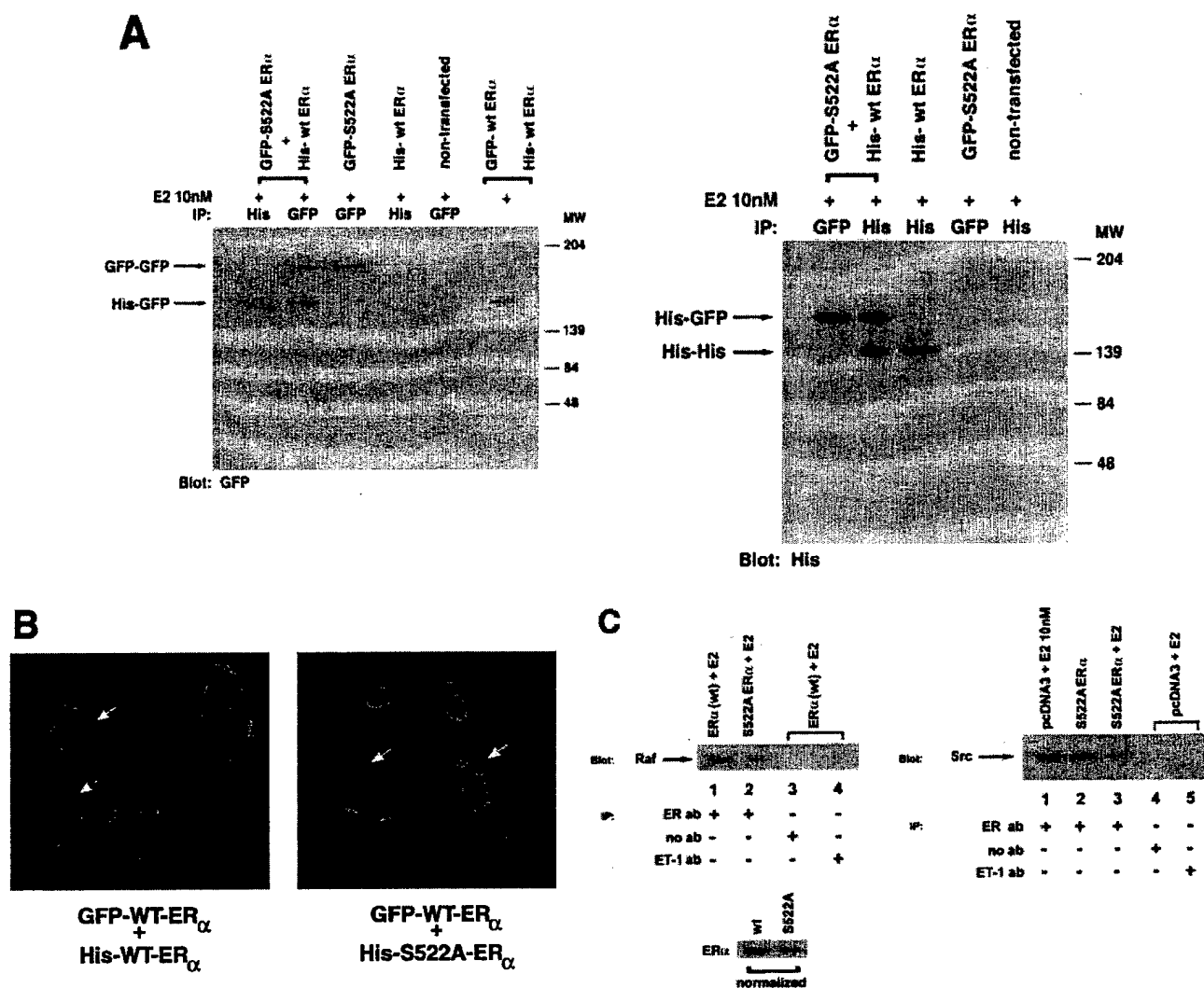


FIG. 7. (A) Homo- and heterodimerization of wt and ER α S522A after expression in CHO cells. Doubly or singly transfected cells were first exposed to 10 nM E2 for 10 min and then lysed, and the lysate underwent immunoprecipitation (IP) with an antibody to His or GFP, as indicated, eventually followed by blotting with an antibody to GFP (left) or to His (right). Proteins were separated by SDS-PAGE under nonreducing conditions (native gel). Molecular weight markers indicate the different sizes of the GFP-tagged wt ER α and His-tagged ER α S522A homodimers and the intermediate size of the heterodimer. (B) Expression of S522A prevents wt ER α localization at the membrane. CHO cells were transfected with equal amounts of plasmids encoding GFP-tagged wt ER α plus His-tagged wt ER α (10 μ g of total DNA/100-mm-diameter dish) or with GFP-tagged wt ER α plus His-tagged ER α S522A. Localization of receptors was determined by confocal microscopy. (C) wt and S522A mutant ER α equally associate with Ras, Raf, or Src at the membrane. (Left) CHO cells were transfected to express either receptor, and after normalization, equal receptor protein aliquots were confirmed by Western blotting. Equal protein aliquots were used for immunoblots to determine wt or mutant ER α association with Ras or Raf. The study was carried out in the presence of 10 nM E2 for 10 min and was repeated. Immunoprecipitation with no antibody (ab) or an irrelevant antibody to the endothelin-1 peptide did not yield a protein band. (Right) MCF-7 cells were transfected with His-tagged ER α S522A (lanes 2 and 3) or with His-tagged pcDNA3 (lane 1) and were incubated or not with 10 nM E2 for 10 min. The cells were lysed and immunoprecipitated for ER α (lane 1) or for His (lanes 2 and 3), then immunoblotted for Src. Expression of His-tagged pcDNA3 did not coprecipitate Src (data not shown).

immunoprecipitations without an antibody or with an irrelevant antibody to endothelin-1 (ET-1) did not bring down a Ras or Raf protein band. Especially important is the association of ER with Src, which has been reported to bind to tyrosine 537 of ER α (24). We examined the effect of expression of ER α S522A on subsequent Src association with ER in MCF-7 cells. As seen in Fig. 7C (right), endogenous ER α associated with Src comparably to the S522A mutant receptor, and this was unaffected by E2. This suggests that there is no alteration of

association with important signaling molecules by ER α S522A that can account for the differences in signaling from the membrane. Thus, we believe that it is primarily the membrane receptor number that determines the differences in signaling.

DISCUSSION

The presence of a PM ER in human cells that enacts signal transduction and thereby contributes to the cellular effects of

E2 is increasingly accepted (14, 33, 34, 40, 49). E2 signaling through PI 3-kinase in vivo rescues muscle from ischemia-reperfusion injury (40), while activation of ERK prevents breast cancer (34) or osteoblast (14) cell death. Recently, Marquez and Pietras have shown that administration of antibodies to ER α in nude mice blocked the growth of human breast cancer xenografts (23). This probably resulted from inhibition by the antibodies of membrane ER signaling to ERK and PI 3-kinase. Spatially, the receptor appears to be localized primarily but not exclusively to caveolar fractions of the PM (6, 13). In this confined area, ER potentially interact with a variety of signaling molecules that must localize to the PM for activation. The PM ER acts as a G protein-coupled receptor, directly (31, 50) or indirectly (10), leading to activation of multiple signaling pathways. This results in cAMP generation (4), PLC and inositol triphosphate (IP3) activation (16, 41), and the stimulation of cascades leading to enhanced activity of ERK, JNK, and p38 MAP kinases (23, 32, 33). The importance of these nongenomic mechanisms of estrogen action is analogous to that of the actions of steroids in plants. In *Arabidopsis* spp., brassinosteroids mediate plant cell developmental growth and fertility (22), and cell action results from steroid binding to a transmembrane, tyrosine kinase receptor protein (44). Thus, steroid action at the cell surface is an ancient function conserved from plants to humans, further indicating its importance.

One important issue with regard to the cell surface ER that we addressed here is the structural requirements for a population of ER to translocate to the PM. We found that ER are not posttranslationally lipidated, as occurs with other PM-localized proteins. Rather, we identified serine 522 as important for membrane translocation. Compared to expression of wt mouse ER α , mutation of this serine to alanine resulted in 62% fewer receptors expressed at the membrane, with little influence on receptor affinity for ligand. However, there was no appreciable effect on the nuclear receptor numbers, affinity, and function (transactivation of an ERE-luciferase reporter). Furthermore, expression of the S522A mutant receptor was markedly less efficient in supporting E2-induced ERK activation, cAMP generation, and stimulation of IP3 than wt receptor expression. Presumably, reduced signaling resulted from a decreased number of receptors available at the membrane. Supporting this, we did not find a loss of association at the membrane between S522A mutant ER α and signaling molecules, compared to that for wt ER α . However, this receptor can serve as a dominant-negative protein for wt ER when expressed in MCF-7 cells, and therefore additional mechanisms of abolishing signal transduction may be relevant (see below). Supporting the specificity of our results, we found that substitution of alanine for serine at residues 10 and 582 of the mouse ER α had no effect on either E2 binding to the membrane or signaling by E2, when these mutants were compared to wt ER α .

How does ER α localize to the membrane, and how does S522 contribute? It was recently determined that caveolin 1 protein facilitates the translocation of ER to the PM and that the two endogenous proteins physically bind in both the cytosol and the PM (31). The scaffolding domain of caveolin 1 (amino acids 82 to 101) is essential for this protein to move from the cytosol to the membrane (3, 27), and we determined that the

scaffolding domain facilitates ER movement to the PM. An important question, then, is whether serine 522 is necessary for the association of ER α and caveolin 1. We report here that in the cytoplasm, the physical association between these two proteins was 60% decreased by the mutation of serine 522. In contrast, association of caveolin with S10- or S582-mutated ER α was comparable to that with wt ER (data not shown). It has recently been shown that residues 1 to 282 of ER α bind to caveolin 1 (37). However, Lu et al. recently showed that caveolin 1 associates with the androgen receptor through both N-terminal (A/B domain) and E domain elements (20). We found that an A/B domain deletion mutant ER α localizes to the membrane and supports E2 signaling to ERK equivalently to wt ER α . Thus, the interaction between caveolin and the N terminus of ER may not be functionally important for the membrane ER.

What supports the relevance of S522A for ER action at the membrane? Kousteni et al. showed that by targeting only the E domain of ER α to the PM but not to the nucleus, E2 could rescue cells from apoptotic death (14). It was recently demonstrated that the E domain (and here the full-length ER) is sufficient to convey robust ERK activation in response to E2 when targeted to the PM (31). These overall findings suggest that the E domain is generally important for ER α actions originating at the membrane. Identification of serine 522 provides a novel insight into the specific structural requirements for membrane localization, steroid action, and cell biological functions of E2. We suggest that similar examination of the role of the ligand binding domains of the progesterone, androgen, and other steroid receptors is warranted.

To establish the roles of the membrane ER in cell biology, several approaches could be taken. Targeting of ER to only one compartment in the cell may suggest a specific function for a pool of the endogenous receptor. Another approach is to devise specific agonists or antagonists for the membrane ER, reagents that do not enter the cell to bind the nuclear receptor. Several ER agonists have recently been described that dissociate some membrane signaling from transcriptional activity (14). However, ER signaling through the membrane receptor stimulates gene transcription (9, 46), and thus, these two functions may not always reflect membrane versus nuclear receptor action. A third approach is to express mutant sex steroid receptors that specifically interfere with endogenous ER actions at the membrane. We show here in MCF-7, ZR-75-1, and endothelial cells that ER α S522A is capable of significantly preventing E2 signaling from the endogenous membrane receptor. We propose that this could result from preventing endogenous ER localization at the membrane. Since the dimerization motifs for ER α do not involve serine 522, we reasoned that wt and mutant ER could heterodimerize and thus sequester wt ER from localizing fully at the PM. Supporting this, we provide evidence of heterodimerization between the mutant and wt ER α and a loss of membrane wt ER α when both receptors are coexpressed.

In MCF-7 or ZR-75-1 breast cancer cells, expression of ER α S522A interfered with endogenous ER function. Expression of ER α S522A inhibited E2-induced ERK activation, cyclin D1 production, cdk4 activity, and G₁/S progression. Many of these actions of E2 require signaling from the membrane to kinases such as ERK. Furthermore, the utility of this approach was

shown in a second cell type, where membrane E2-ER signaling to p38 β MAP kinase (33) was significantly prevented by expression of ER α S522A. The strong inhibition of cyclin D1 protein in MCF-7 cells by ER α S522A expression and the linkage to modulation of ERK activity suggests an important therapeutic intervention in breast cancer. In vitro, E2 induction of cyclin D1 overcomes the tamoxifen-induced G₁/S cell cycle block (47). Also, tamoxifen sensitivity can be restored through p27 function, resulting from ERK downregulation (8). In this respect, limiting endogenous membrane ER signaling to ERK (19) and cyclin D1 may be therapeutically desirable, as suggested by our use of the S522A mutant ER α . It has also been recently reported that specifically cyclin D1 is essential to the development of rodent breast cancer, resulting from Ras or Neu oncogene signaling (51). Cyclin D1 has several important functions, but arguably the most important is the regulation of the inactivating phosphorylation of the Rb protein by cdk4, allowing E2F release and the subsequent transcription of genes that drive cell cycle progression in breast cancer (39). Our demonstration that ER α S522A significantly limits these events both points out therapeutic targets and reveals the importance of E2 signaling from the membrane. The ultimate goal of hormone replacement after the menopause is to activate specific, desirable effects of sex steroids (osteoblast survival) without invoking unwanted actions (breast cancer proliferation). This strategy will be best served by defining the array of discrete actions of E2 that result from binding at membrane and nuclear ER in various target cells. Expression of ER α S522A may be very useful in this regard.

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REFERENCES

- Albanese, C., J. Johnson, G. Watanabe, N. Eklund, D. Vu, A. Arnold, and R. G. Pestell. 1995. Transforming p21 ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. *J. Biol. Chem.* 270: 23589–23597.
- Alton, G., M. Hasilik, R. Niehues, K. Paneriselvam, J. R. Etchison, F. Fana, and H. H. Freeze. 1998. Direct utilization of mannose for mammalian glycoprotein biosynthesis. *Glycobiology* 8:285–295.
- Anderson, R. G. 1998. The caveolae membrane system. *Annu. Rev. Biochem.* 67:199–225.
- Aronica, S. M., W. L. Kraus, and B. S. Katzenellenbogen. 1994. Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. *Proc. Natl. Acad. Sci. USA* 91:8517–8521.
- Catoria, G., M. V. Barone, M. Di Domenico, A. Bilancio, D. Ametrano, A. Migliaccio, and F. Auricchio. 1999. Non-transcriptional action of oestradiol and progestin triggers DNA synthesis. *EMBO J.* 18:2500–2510.
- Chambliss, K. L., I. S. Yuhanna, C. Mineo, P. Liu, Z. German, T. S. Sherman, M. E. Mendelsohn, R. G. W. Anderson, and P. W. Shaul. 2000. Estrogen receptor α and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae. *Circ. Res.* 87:e44–e52.
- Cooper, L. F., J. C. Tiffie, J. P. Griffin, H. Hamano, and Z. Guo. 2000. Estrogen-induced resistance to osteoblast apoptosis is associated with increased hsp27 expression. *J. Cell. Physiol.* 185:401–407.
- Donovan, J. C. H., A. Milic, and J. M. Slingerland. 2001. Constitutive MEK/MAPK activation leads to p27kip1 deregulation and antiestrogen resistance in human breast cancer cells. *J. Biol. Chem.* 276:40888–40895.
- Duan, R., W. Xie, and S. Safe. 2001. Estrogen receptor-mediated activation of the serum response element in MCF-7 cells through MAPK-dependent phosphorylation of Elk-1. *J. Biol. Chem.* 276:11590–11598.
- Fitardo, E. J., J. A. Quinn, K. I. Bland, and R. A. Frackelton, Jr. 2000. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, gpr30, and occurs via transactivation of the epidermal growth factor receptor through release of HB-EGF. *Mol. Endocrinol.* 14:1649–1660.
- Greene, G., N. Sobel, W. King, and E. Jensen. 1984. Immunochemical studies of estrogen receptors. *J. Steroid Biochem.* 20:51–56.
- Holt, A., G. Alton, C. H. Scaman, G. Loppnow, A. Szpacenko, I. Svendsen, and M. M. Palcic. 1998. Identification of the quinone growth cofactor in mammalian semicarbazide-sensitive amine oxidase. *Biochemistry* 37:4946–4957.
- Kim, H. P., J. Y. Lee, J. K. Jeong, S. W. Bae, H. K. Lee, and I. Jo. 1999. Nongenomic stimulation of nitric oxide release by estrogen is mediated by estrogen receptor α localized in caveolae. *Biochem. Biophys. Res. Commun.* 263:257–262.
- Kousteni, S., T. Bellido, L. I. Plotkin, C. A. O'Brien, D. L. Bodenner, L. Han, K. Han, G. B. DiGregorio, J. A. Katzenellenbogen, B. S. Katzenellenbogen, P. K. Roberson, R. S. Weinstein, R. L. Jilka, and S. C. Manolagas. 2001. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell* 104:719–730.
- Lavoie, J. N., G. L'Allemain, A. Brunet, R. Muller, and J. Pouyssegur. 1996. Cyclin D1 expression is regulated by the p42/p44 MAPK and negatively by the p38/HOG MAPK pathway. *J. Biol. Chem.* 271:20608–20616.
- Le Mellay, V., B. Grosse, and M. Lieberherr. 1997. Phospholipase C β and membrane action of calcitriol and estradiol. *J. Biol. Chem.* 272:11902–11907.
- Levin, E. R. 1999. Cellular functions of the plasma membrane estrogen receptor. *Trends Endocrinol. Metab.* 10:374–376.
- Lieberherr, M., B. Grosse, M. Kachkache, and S. Balsan. 1993. Cell signaling and estrogens in female rat osteoblasts: a possible involvement of unconventional non-nuclear receptors. *J. Bone Min. Res.* 8:1365–1376.
- Lobenhofer, E. K., G. Huper, J. D. Iglehart, and J. R. Marks. 2000. Inhibition of mitogen-activated protein kinase and phosphatidylinositol 3-kinase activity in MCF-7 cells prevents estrogen-induced mitogenesis. *Cell Growth Differ.* 11:99–110.
- Lu, M. L., M. C. Schneider, Y. Zhang, and J. P. Richie. 2001. Caveolin-1 interacts with androgen receptor: a positive modulator of androgen receptor mediated transactivation. *J. Biol. Chem.* 276:13442–13451.
- Lublin, D. 1992. Glucosyl-phosphatidylinositol anchoring of membrane proteins. *Curr. Top. Microbiol. Immunol.* 178:142–168.
- Mandava, M. B. 1988. Plant growth-promoting brassinosteroids. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 39:23–52.
- Marquez, D. C., and R. J. Pietras. 2001. Membrane-associated binding sites for estrogen contribute to growth regulation of human breast cancer cells. *Oncogene* 20:5420–5430.
- Migliaccio, A., M. Di Domenico, G. Castoria, A. de Falco, P. Bontempo, E. Nola, and F. Auricchio. 1996. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *EMBO J.* 15:1292–1300.
- Morey, A. K., A. Pedram, M. Razandi, B. A. Prins, R.-M. Hu, E. Biesiada, and E. R. Levin. 1997. Estrogen and progesterone inhibit human vascular smooth muscle proliferation. *Endocrinology* 138:3330–3339.
- Norfleet, A. M., M. L. Thomas, B. Gametchu, and C. S. Watson. 1999. Estrogen receptor- α detected on the plasma membrane of aldehyde-fixed GH3/B6/F10 rat pituitary tumor cells by enzyme-linked immunocytochemistry. *Endocrinology* 140:3805–3814.
- Okamoto, T., A. Schlegel, P. E. Scherer, and M. P. Lisanti. 1998. Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. *J. Biol. Chem.* 273:5419–5422.
- Pedram, A., M. Razandi, and E. R. Levin. 1998. Extracellular regulated kinase/jun kinase cross-talk underlies vascular endothelial cell growth factor-induced endothelial cell proliferation. *J. Biol. Chem.* 273:26722–26728.
- Pettersson, K., F. Delaunay, and J. A. Gustafsson. 2000. Estrogen receptor β acts as a dominant regulator of estrogen signaling. *Oncogene* 19:4970–4978.
- Pietras, R., and C. M. Szego. 1977. Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. *Nature* 265:69–72.
- Razandi, M., P. Oh, A. Pedram, J. Schnitzer, and E. R. Levin. 2002. Estrogen receptors associate with and regulate the production of caveolin: implications for signaling and cellular actions. *Mol. Endocrinol.* 16:100–115.
- Razandi, M., A. Pedram, G. L. Greene, and E. R. Levin. 1999. Cell membrane and nuclear estrogen receptors derive from a single transcript: studies of ER α and ER β expressed in CHO cells. *Mol. Endocrinol.* 13:307–319.
- Razandi, M., A. Pedram, and E. R. Levin. 2000. Estrogen signals to preservation of endothelial cell form and function. *J. Biol. Chem.* 275:38540–38546.
- Razandi, M., A. Pedram, and E. R. Levin. 2000. Plasma membrane estrogen receptors signal to anti-apoptosis in breast cancer. *Mol. Endocrinol.* 14: 1434–1447.
- Resh, M. D. 1996. Regulation of cellular signalling by fatty acid acylation and prenylation of signal transduction proteins. *Cell. Signal.* 8:403–412.
- Russell, K. S., M. P. Haynes, D. Sinha, E. Clerisme, and J. R. Bender. 2000. Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. *Proc. Natl. Acad. Sci. USA* 97:5930–5935.

37. Schlegel, A., C. Wang, R. G. Pestell, and M. P. Lisanti. 2001. Ligand-independent activation of oestrogen receptor α by caveolin-1. *Biochem. J.* 359:203–210.
38. Schnitzer, J. E., D. P. McIntosh, A. M. Dvorak, J. Liu, and P. Oh. 1995. Separation of caveolae from associated microdomains of GPI-anchored proteins. *Science* 269:1435–1439.
39. Sherr, C. J. 1993. Mammalian G₁ cyclins. *Cell* 73:1059–1065.
40. Simoncini, T., A. Hafezi-Moghadam, D. P. Brazil, K. Ley, W. W. Chin, and J. K. Liao. 2000. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* 407:538–541.
41. Tesarik, J., and C. Mendoza. 1995. Nongenomic effects of 17 β -estradiol on maturing human oocytes: relationship to oocyte developmental potential. *J. Clin. Endocrinol. Metab.* 80:1438–1443.
42. Truss, M., and M. Beato. 1993. Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. *Endocrine Rev.* 14:459–479.
43. Valentine, J. E., E. Kalkhoven, R. White, S. Hoare, and M. G. Parker. 2000. Mutations in the estrogen receptor ligand binding domain discriminate between hormone-dependent transactivation and transrepression. *J. Biol. Chem.* 275:25322–25329.
44. Wang, Z.-Y., H. Seto, S. Fujioka, S. Yoshida, and J. Chory. 2001. BR11 is a critical component of a plasma-membrane receptor for plant steroids. *Nature* 410:380–383.
45. Watters, J. J., J. S. Campbell, M. J. Cunningham, E. G. Krebs, and D. M. Dorsa. 1997. Rapid membrane effects of steroids in neuroblastoma cells: effects of estrogen on mitogen activated protein kinase signalling cascade and c-fos immediate early gene transcription. *Endocrinology* 138:4030–4033.
46. Watters, J. J., T. Y. Chun, Y. N. Kim, P. J. Bertics, and J. Gorski. 2000. Estrogen modulation of prolactin gene expression requires an intact mitogen-activated protein kinase signal transduction pathway in cultured rat pituitary cells. *Mol. Endocrinol.* 14:1872–1881.
47. Watts, C. K., K. J. Sweeney, A. Warlters, E. A. Musgrove, and R. L. Sutherland. 1994. Antiestrogen regulation of cell cycle progression and cyclin D1 gene expression in MCF-7 human breast cancer cells. *Breast Canc. Res. Treat.* 31:95–105.
48. White, R., J. A. Lees, M. Needham, J. Ham, and M. Parker. 1987. Structural organization and expression of the mouse estrogen receptor. *Mol. Endocrinol.* 1:735–744.
49. Wise, P. M., D. B. Dubal, M. E. Wislon, S. W. Rau, and M. Bottner. 2001. Neuroprotective effects of estrogen—new insights into mechanisms of action. *Endocrinology* 142:969–973.
50. Wyckoff, M. H., K. L. Chambliss, C. Mineo, I. S. Yuhanna, M. E. Mendelsohn, S. M. Mumby, and P. W. Shaul. 2001. Plasma membrane estrogen receptors are coupled to endothelial nitric-oxide synthase through G α_q . *J. Biol. Chem.* 276:27071–27076.
51. Yu, Q., Y. Geng, and P. Sicinski. 2001. Specific protection against breast cancers by cyclin D1 ablation. *Nature* 411:1017–1021.