

# Direct Interactions with G $\alpha$ i and G $\beta$ $\gamma$ Mediate Nongenomic Signaling by Estrogen Receptor $\alpha$

Premlata Kumar, Qian Wu, Ken L. Chambliss, Ivan S. Yuhanna, Susanne M. Mumby, Chieko Mineo, Gregory G. Tall, and Philip W. Shaul

*Departments of Pediatrics (P.K., Q.W., K.L.C., I.S.Y., C.M., P.W.S.) and Pharmacology (S.M.M., G.G.T.), University of Texas Southwestern Medical Center, Dallas, Texas 75390*

Estrogen induces G protein-dependent nongenomic signaling in a variety of cell types via the activation of a plasma membrane-associated subpopulation of estrogen receptor  $\alpha$  (ER $\alpha$ ). Using pull-down experiments with purified recombinant proteins, we now demonstrate that ER $\alpha$  binds directly to G $\alpha$ i and G $\beta$  $\gamma$ . Mutagenesis and the addition of blocking peptide reveals that this occurs via amino acids 251–260 and 271–595 of ER $\alpha$ , respectively. Studies of ER $\alpha$  complexed with heterotrimeric G proteins further show that estradiol causes the release of both G $\alpha$ i and G $\beta$  $\gamma$  without stimulating GTP binding to G $\alpha$ i. Moreover, in COS-7 cells, the disruption of ER $\alpha$ -G $\alpha$ i interaction

by deletion mutagenesis of ER $\alpha$  or expression of blocking peptide, as well as G $\beta$  $\gamma$  sequestration with  $\beta$ -adrenergic receptor kinase C terminus, prevents nongenomic responses to estradiol including src and erk activation. In endothelial cells, the disruption of ER $\alpha$ -G $\alpha$ i interaction prevents estradiol-induced nitric oxide synthase activation and the resulting attenuation of monocyte adhesion that contributes to estrogen-related cardiovascular protection. Thus, through direct interactions, ER $\alpha$  mediates a novel mechanism of G protein activation that provides greater diversity of function of both the steroid hormone receptor and G proteins. (*Molecular Endocrinology* 21: 1370–1380, 2007)

**S**TEROID HORMONE RECEPTORS (SHRs) function classically in the nucleus as ligand-activated transcription factors. More recently, it has become apparent that steroid hormones also initiate a diverse set of important nongenomic cellular responses via the activation of plasma membrane-associated SHRs (1, 2). In particular, this has been elucidated regarding the nonnuclear actions of estrogen, which modify growth and differentiation, migration, and other processes in cell types as diverse as oocytes, osteoblasts, osteoclasts, neurons, breast cancer cells, adipocytes, and endothelial cells (1–5). The underlying mechanisms are best exemplified by the identification of a caveolae membrane-associated population of the classical estrogen receptor  $\alpha$  (ER $\alpha$ ) in endothelial cells that activates Src family tyrosine kinases, phosphatidylinositol 3 kinase/Akt kinase, and erk1,2 to stimulate nitric oxide (NO) production by the endothelial isoform of NO synthase (eNOS). These pathways are critically involved in estrogen-related cardiovascular protection (6). Activation of these pathways also stimulates phos-

phorylation of ER $\alpha$  and its coregulators and S-nitrosylation of the receptor to modify nuclear signaling, indicating that there is additional important cross talk between membrane and nuclear SHR function (3, 7).

In previous studies using endothelial cells, we demonstrated that signal initiation by membrane ER $\alpha$  is pertussis toxin (PTX) sensitive and that ER $\alpha$  and G $\alpha$ i can be coimmunoprecipitated from the plasma membrane (8). These findings and related evidence of heterotrimeric G protein involvement in signaling by ER in other cell types (9) raise the possibility that the most proximal mechanisms underlying membrane SHR actions entail interactions with G proteins. Heterotrimeric G proteins are activated conventionally by members of a family of G protein-coupled receptors (GPCRs), the sequences of which predict structures of seven membrane spans that include binding sites for G proteins. Agonist binding to GPCRs promotes the release of GDP from G $\alpha$ , thus allowing G $\alpha$  to bind the more abundant nucleotide in the cell, GTP. A conformational change in G $\alpha$  accompanies GTP binding, leading to the dissociation of G $\alpha$  and the high-affinity complex of  $\beta$  and  $\gamma$  subunits from the GPCR. Liberated G $\alpha$ -GTP and  $\beta$  $\gamma$  subunits are competent to modulate the activity of downstream effectors (10, 11). In contrast to the in-depth knowledge available regarding G protein and GPCR interactions, the molecular basis of the functional linkage between SHRs such as ER $\alpha$  and G proteins is unknown.

In the present investigation we designed experiments to test the hypothesis that ER $\alpha$  interacts directly with G $\alpha$ i. Further studies were performed to address the following questions: 1) Are interactions between

## First Published Online April 3, 2007

Abbreviations: AR, Androgen receptor;  $\beta$ ARK-ct,  $\beta$ -adrenergic receptor kinase C-terminal tail; BAEC, bovine aortic endothelial cell; eNOS, endothelial isoform of nitric oxide synthase; ER, estrogen receptor; GPCR, G protein-coupled receptor; GR, glucocorticoid receptor; LPS, lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase; NTA, nitrilotriacetic acid; PTX, pertussis toxin; SHR, steroid hormone receptor; VDR, vitamin D receptor.

***Molecular Endocrinology* is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.**

ER $\alpha$  and G $\alpha$ i required for nongenomic signaling by the receptor? 2) Do other SHR that mediate membrane-initiated signaling interact directly with G $\alpha$ i? 3) Does ER $\alpha$  also interact directly with G $\beta\gamma$ ? 4) What are the domains of ER $\alpha$  that interact with G $\alpha$ i and G $\beta\gamma$ ? 5) How does the interaction of ER $\alpha$  with G proteins initiate signaling? and 6) Do these mechanisms modify the function of endothelial cells, which have well-recognized nongenomic responses to estrogen of importance to cardiovascular protection?

## RESULTS

### ER $\alpha$ Interaction with G $\alpha$ i

To first investigate whether ER $\alpha$  interacts directly with monomeric G $\alpha$ i, we performed pull-down experiments using purified myristoylated G $\alpha$ i-GDP that contained a hexahistidine tag inserted at amino acid position 121 to preserve myristoylation and typical receptor interactions with the G $\alpha$ i C terminus (His<sub>6</sub>-G $\alpha$ i-GDP) (12, 13). Direct protein-protein interactions were evaluated with recombinant ER $\alpha$  protein in the absence or presence of varying concentrations of 17 $\beta$ -estradiol (E<sub>2</sub>). In the absence of ligand, ER $\alpha$  bound G $\alpha$ i and the interaction was enhanced by E<sub>2</sub> in a dose-dependent manner (Fig. 1A). The addition of ICI 182,780 alone blunted the interaction, and it also attenuated the enhancement in interaction prompted by E<sub>2</sub> (supplemental Fig. 1A, published as supplemental data on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>). In contrast to E<sub>2</sub>, dexamethasone and dihydrotestosterone had no effect on the ER $\alpha$ -G $\alpha$ i interaction (data not shown). ER $\alpha$  interacted preferentially with GDP-bound *vs.* GTP $\gamma$ S-bound G $\alpha$ i (Fig. 1B), and the interaction was enhanced by G $\alpha$ i myristoylation (Fig. 1C).

To determine whether G protein interaction with ER $\alpha$  mediates nongenomic responses to E<sub>2</sub>, we evaluated src activation in COS-7 cells expressing ER $\alpha$  in the presence or absence of PTX. Src activation is an early signaling event in multiple membrane-initiated actions of E<sub>2</sub> and ER $\alpha$  (1–5), numerous known GPCRs control src activation (14, 15), and we have previously shown that PTX prevents ligand-dependent ER $\alpha$ -G $\alpha$ i coimmunoprecipitation from plasma membranes (8). E<sub>2</sub> (10<sup>-8</sup> M)-induced src phosphorylation was prevented by PTX treatment (Fig. 1, D and E), indicating that G $\alpha$ i interaction with ER $\alpha$  is a required proximal process in membrane ER $\alpha$  signaling.

### Interactions between Other SHRs and G $\alpha$ i

Multiple SHRs in addition to ER $\alpha$  initiate rapid responses upon ligand activation that are independent of the modification of gene transcription (1, 2, 16). To investigate whether the direct interaction observed between ER $\alpha$  and G $\alpha$ i is shared by other SHRs for which there is evidence of nongenomic signaling involving G proteins (1, 2, 17–19), plasma membranes purified

from COS-7 cells expressing ER $\beta$ , androgen receptor (AR), glucocorticoid receptor (GR), or vitamin D receptor (VDR) were tested in the myristoylated His<sub>6</sub>-G $\alpha$ i-GDP pull-down assay. Membrane-associated ER $\beta$  and AR bound G $\alpha$ i, and binding was enhanced by the relevant SHR ligand (Fig. 2, A and B). In contrast, GR and VDR did not bind to His<sub>6</sub>-G $\alpha$ i-GDP in the absence or presence of ligand (Fig. 2, C and D). To determine whether the membrane-associated ER $\beta$  and AR interactions with G $\alpha$ i signify direct protein-protein binding, additional His<sub>6</sub>-G $\alpha$ i-GDP pull-down experiments were performed with purified recombinant receptor proteins. Recombinant ER $\beta$  bound G $\alpha$ i, and the interaction was enhanced by E<sub>2</sub> (Fig. 2E). Recombinant AR in truncated form (amino acids 606–902) also bound G $\alpha$ i, and there was increased interaction in the presence of dihydrotestosterone (Fig. 2F). Thus, direct interaction with G $\alpha$ i is a shared feature of ER $\alpha$  and select SHRs that initiate signaling at the plasma membrane.

### Domain of ER $\alpha$ Mediating Interaction with G $\alpha$ i

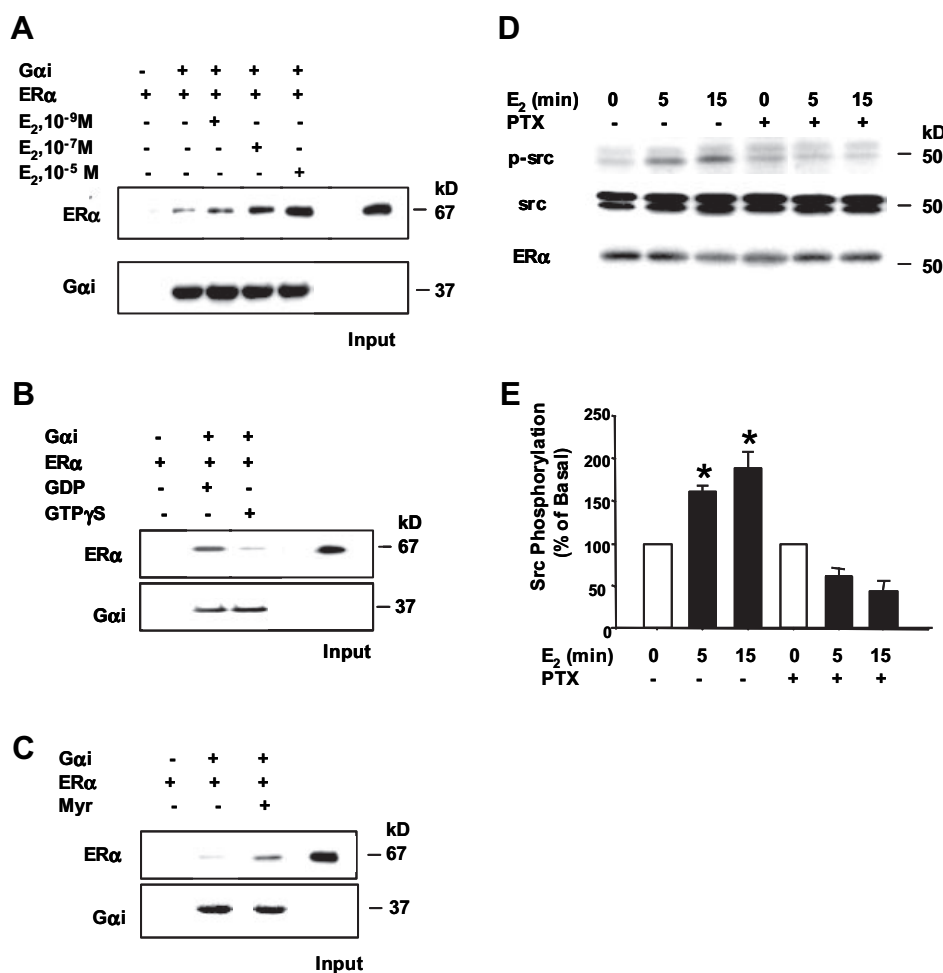
To identify the domain of ER $\alpha$  involved in direct interaction with G $\alpha$ i, Flag-tagged wild-type and deletion mutant human ER $\alpha$  proteins (Fig. 3A) were expressed and purified from baculovirus-infected Sf9 cells and used in pull-down assays with myristoylated His<sub>6</sub>-G $\alpha$ i-GDP. Wild-type ER $\alpha$  and an N-terminal deletion mutant lacking amino acids 1–175 (ER $\alpha$  $\Delta$ 1–175) bound G $\alpha$ i comparably (Fig. 3B). A C-terminal deletion mutant lacking amino acids 271–595 (ER $\alpha$  $\Delta$ 271–595) also interacted with G $\alpha$ i, but an internal deletion of amino acids 180–268 (ER $\alpha$  $\Delta$ 180–268) prevented binding with G $\alpha$ i (Fig. 3C). In contrast to ER $\alpha$  $\Delta$ 180–268, a mutant receptor lacking amino acids 185–251 (ER $\alpha$  $\Delta$ 185–251) was capable of interaction with G $\alpha$ i (Fig. 3D), implicating amino acids 180–184 and/or 252–268 in binding with the G protein. Further investigation revealed that G $\alpha$ i interaction does not involve amino acids 261–271, and instead it was found that the region of ER $\alpha$  between amino acids 250 and 260 mediates direct binding to G $\alpha$ i (Fig. 3E). Moreover, the introduction of a peptide representing amino acids 251–260 of ER $\alpha$  disrupted the interaction between the wild-type receptor and G $\alpha$ i, whereas scrambled peptide did not (Fig. 3F), confirming the findings made by deletion mutagenesis.

The involvement of G $\alpha$ i binding to amino acids 250–260 of ER $\alpha$  in nongenomic receptor signaling was then evaluated in studies of src phosphorylation in COS-7 cells. Whereas wild-type ER $\alpha$  promoted src phosphorylation with E<sub>2</sub>, no response was evident in cells expressing ER $\alpha$  $\Delta$ 250–260 (Fig. 4, A and B). In parallel, we found that expression of an HA-tagged peptide consisting of only amino acids 251–260 of ER $\alpha$  mimicked the action of PTX (Fig. 1, D and E) and blocked nongenomic signaling by wild-type ER $\alpha$  to src (Fig. 4, C and D). These collective observations identify amino acids 251–260 of ER $\alpha$  as a G $\alpha$ i binding domain that is critically involved in nongenomic signaling by the receptor.

### ER $\alpha$ Interaction with Heterotrimeric G $\alpha\beta\gamma$ and G $\beta\gamma$ Dimer

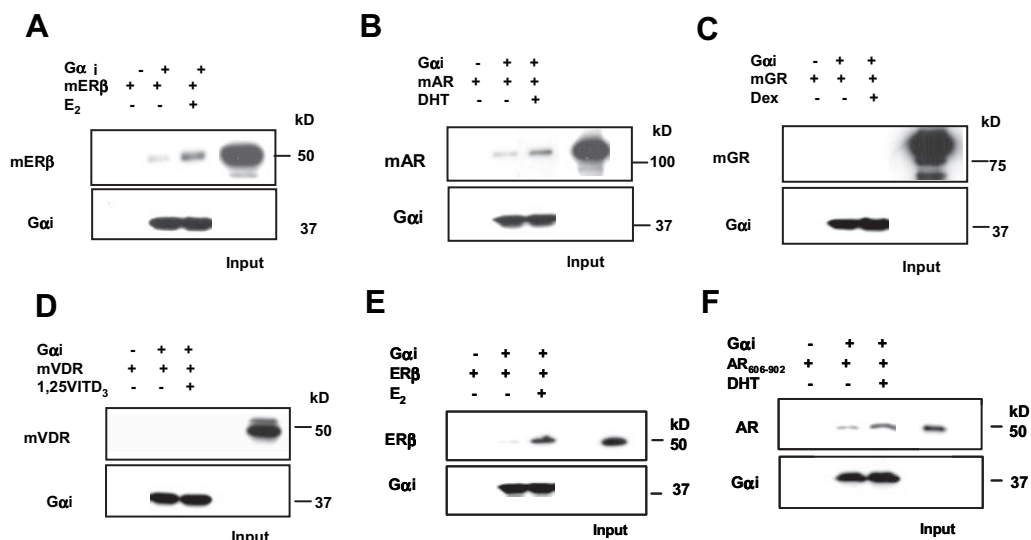
In order for G $\alpha$ i to interact effectively with classical GPCRs, G $\alpha$ i is associated with G $\beta\gamma$  as a heterotrimer. In addition, both activated G $\alpha$ i and G $\beta\gamma$  are capable of modulating the activity of downstream effector molecules (10, 11). Therefore, pull-down experiments were performed using purified components to compare the capacity of ER $\alpha$  to bind monomeric His $_6$ -G $\alpha$ i-GDP and His $_6$ -G $\alpha$ i-GDP associated with G $\beta_1\gamma_2$ . In the absence of agonist, ER $\alpha$  interaction with G $\alpha$ i was enhanced by G $\beta\gamma$  (Fig. 5A). Whereas ER $\alpha$  interaction with monomeric G $\alpha$ i was increased by E $_2$ , the addition of the ligand diminished the interaction between the receptor and G $\alpha$ i in

heterotrimeric form. These observations raised the possibility that ER $\alpha$  also binds G $\beta\gamma$  directly. Pull-down experiments employing Flag-tagged ER $\alpha$  demonstrated that such an interaction occurs, and that it is attenuated by E $_2$  (Fig. 5B). The addition of ICI 182,780 reversed the E $_2$ -induced decrease in interaction between ER $\alpha$  and G $\beta\gamma$ , and ICI 182,780 alone actually caused an increase in the interaction (supplemental Fig. 1B, published as supplemental data on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>). Furthermore, a decline in the ER $\alpha$ -G $\beta_1\gamma_2$  interaction, and also in the ER $\alpha$ -G $\alpha$ i interaction, occurred with the addition of E $_2$  to the Flag-tagged ER $\alpha$  pull-down of G protein heterotrimer (Fig. 5C). Moreover, in heterotrimer experiments ICI reversed the loss of ER $\alpha$ -G $\alpha$ i interaction that



**Fig. 1.** ER $\alpha$  Interacts Directly with G $\alpha$ i, and the Interaction Is Required for Signaling to src

A, Pull-down experiments were performed with myristoylated His $_6$ -G $\alpha$ i-GDP and recombinant ER $\alpha$  in the absence or presence of E $_2$  at the indicated concentrations. Immunoblot analyses were performed for ER $\alpha$  and G $\alpha$ i. In all pull-down experiments, the input lanes represent 20% of the amount of protein used and the sample lanes contain 50% of the pull-downs. Results shown for all pull-downs are representative of three or more independent studies. B, Pull-downs were performed with myristoylated GDP- vs. GTP $\gamma$ S-bound His $_6$ -G $\alpha$ i. C, Pull-downs were performed with nonmyristoylated vs. myristoylated His $_6$ -G $\alpha$ i-GDP. D, COS-7 cells expressing ER $\alpha$  were pretreated with vehicle or PTX (100 ng/ml for 120 min) and incubated with 10<sup>-8</sup> M E $_2$  for 0–15 min, and src activation was evaluated by immunoblot analyses of cell lysates using anti-phospho-src (p-src) polyclonal and anti-src monoclonal antibodies. E, Cumulative results for src phosphorylation expressed as percentage in nontreated cells (basal) for three independent studies (mean  $\pm$  SEM; \*,  $P < 0.05$  vs. basal).



**Fig. 2.** G $\alpha$ i Interacts with a Distinct Subset of SHRs

Purified myristoylated His<sub>6</sub>-G $\alpha$ i-GDP was used to pull down plasma membrane-associated (designated “m”) ER $\beta$  (A), AR (B), GR (C), and VDR (D) in the absence or presence of vehicle or 10<sup>-5</sup> M E<sub>2</sub>, dihydrotestosterone, dexamethasone, or 1,25-dihydroxy vitamin D<sub>3</sub>, respectively. Proteins were eluted from the Ni-NTA resin with sample buffer and immunoblot analyses were performed for ER $\beta$ , AR, GR, VDR, and G $\alpha$ i. In all experiments, the input lanes represent 20% of the amount of protein employed in the pull-down, and the sample lanes contain 50% of the pull-down. E and F, Pull-down experiments were performed with myristoylated His<sub>6</sub>-G $\alpha$ i-GDP and either recombinant ER $\beta$ , in the absence or presence of 10<sup>-5</sup> M E<sub>2</sub> (E), or recombinant AR<sub>606-902</sub> in the absence or presence of 10<sup>-5</sup> M dihydrotestosterone (DHT) (F). All results shown are representative of three independent studies.

occurs with E<sub>2</sub>, but not the decrease in ER $\alpha$ -G $\beta$  $\gamma$  interaction with E<sub>2</sub> (supplemental Fig. 1C, published as supplemental data on The Endocrine Society’s Journals Online web site at <http://mend.endojournals.org>); in fact, ICI 182,780 alone acted similar to E<sub>2</sub> alone and caused a decline in ER $\alpha$ -G $\beta$  $\gamma$  interaction. Thus, the modulation of ER $\alpha$ -G protein interactions by the ER $\alpha$  ligands E<sub>2</sub> and ICI 182,780 differs whether or not the G proteins are in heterotrimeric form.

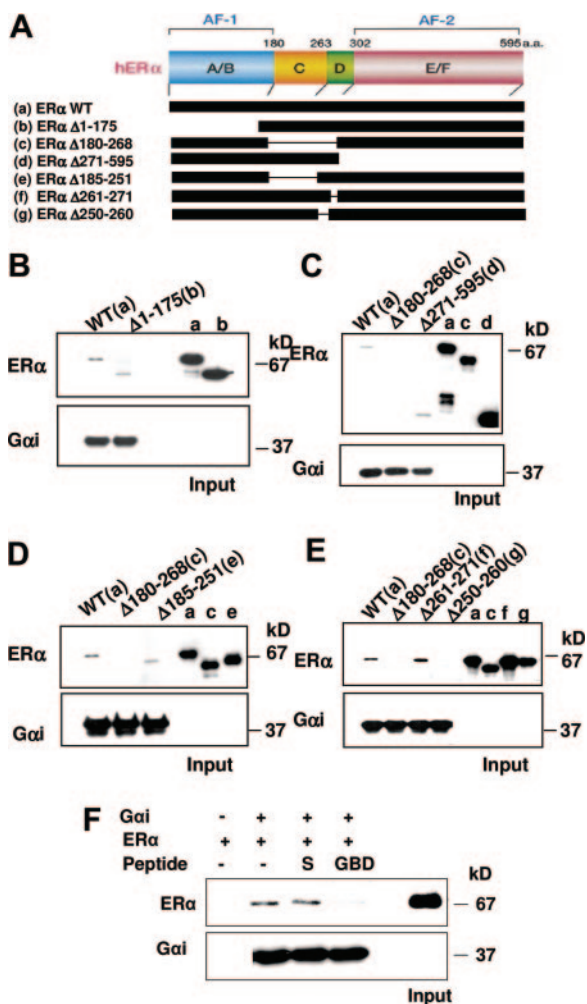
To identify the domain(s) of ER $\alpha$  involved in direct G $\beta$  $\gamma$  binding, experiments were performed using Flag-tagged wild-type ER $\alpha$  and deletion mutants of ER $\alpha$  to pull down G $\beta$  $\gamma$ . Whereas wild-type ER $\alpha$  and the deletion mutant lacking amino acids 180–268 (ER $\alpha$  $\Delta$ 180–268) displayed comparable binding with G $\beta$  $\gamma$ , the mutant lacking amino acids 271–595 (ER $\alpha$  $\Delta$ 271–595) did not interact with G $\beta$  $\gamma$  (Fig. 5D). Thus, G $\beta$  $\gamma$  interacts directly with ER $\alpha$  via a receptor domain(s) that is distinct from the G $\alpha$ i binding domain. Moreover, the interaction with G $\beta$  $\gamma$  promotes receptor interaction with G $\alpha$ i, and the complex formed by the receptor and heterotrimeric G $\alpha$  $\beta$  $\gamma$  is disrupted upon agonist binding to the receptor.

### Mechanism Underlying Signal Initiation by ER $\alpha$ Complexed with G $\alpha$ $\beta$ $\gamma$

Having observed that the ER $\alpha$  interaction with G $\alpha$ i is required for signal transduction and that the receptor also complexes G $\alpha$  $\beta$  $\gamma$  directly, the potential ability of ER $\alpha$  to activate G protein heterotrimers by acting as a

guanine nucleotide exchange catalyst was explored. Membranes prepared from Sf9 cells coexpressing heterotrimeric G $\alpha$  $\beta$  $\gamma$  and either ER $\alpha$  or the M2 muscarinic receptor were incubated with [<sup>35</sup>S]GTP $\gamma$ S to determine the kinetics of G $\alpha$ i nucleotide binding. In the absence of ligand, the M2 muscarinic receptor did not alter the rate of G $\alpha$ i-GTP production appreciably (Fig. 6A); in contrast, expression of ER $\alpha$  appeared to stimulate a slow kinetic exchange of guanine nucleotide (Fig. 6B). However, whereas carbachol stimulation of the M2 muscarinic receptor promoted more rapid G $\alpha$ i [<sup>35</sup>S]GTP $\gamma$ S binding (Fig. 6A), E<sub>2</sub> stimulation of ER $\alpha$  did not result in an increase in [<sup>35</sup>S]GTP $\gamma$ S binding (Fig. 6B), and ICI 182,780 also did not affect guanine nucleotide exchange (data not shown). These results suggest that the mechanism of E<sub>2</sub>-induced activation of ER $\alpha$  and G proteins is more complex than simple regulation of the G $\alpha$ i guanine nucleotide switch, thus differing significantly from GPCR-induced signaling.

Because we demonstrated that G $\beta$  $\gamma$  interacts dynamically with ER $\alpha$  and free G $\beta$  $\gamma$  can mediate subsequent cellular responses in diverse paradigms (10, 11), the possibility that liberated G $\beta$  $\gamma$  modulates downstream signaling by ER $\alpha$  was investigated. COS-7 cells expressing ER $\alpha$  were transfected with either empty plasmid or plasmid encoding the  $\beta$ -adrenergic receptor kinase C-terminal tail ( $\beta$ ARK-ct). Under control conditions, E<sub>2</sub> stimulated the phosphorylation of src (Fig. 6, C and D). In contrast, in cells expressing  $\beta$ ARK-ct, there was an abrogation of E<sub>2</sub>-induced src activation. To evaluate an-



**Fig. 3.** Amino Acids 251–260 of ER $\alpha$  Interact Directly with G $\alpha$ i

A, Schematic of Flag-tagged wild-type ER $\alpha$  (WT) and ER $\alpha$  deletion mutants used in myristoylated His<sub>6</sub>-G $\alpha$ i-GDP pull-down experiments. The identities of the constructs are designated by lowercase letters on the schematic (A) and in the results shown (B–E). Comparisons were made in simultaneous pull-downs with WT ER $\alpha$  vs. ER $\alpha$  $\Delta$ 1–175 (B), WT ER $\alpha$  vs. ER $\alpha$  $\Delta$ 180–268 or ER $\alpha$  $\Delta$ 271–595 (C), WT ER $\alpha$  vs. ER $\alpha$  $\Delta$ 180–268 or ER $\alpha$  $\Delta$ 185–251 (D), or with WT ER $\alpha$  vs. ER $\alpha$  $\Delta$ 180–268, ER $\alpha$  $\Delta$ 261–271, and ER $\alpha$  $\Delta$ 250–260 (E). The interaction between WT ER $\alpha$  and G $\alpha$ i was also tested in the absence or presence of a peptide representing the G $\alpha$ i binding domain (GBD) comprised of amino acids 251–260 of the receptor or scrambled peptide (S) (F). Results shown are representative of three or more independent studies.

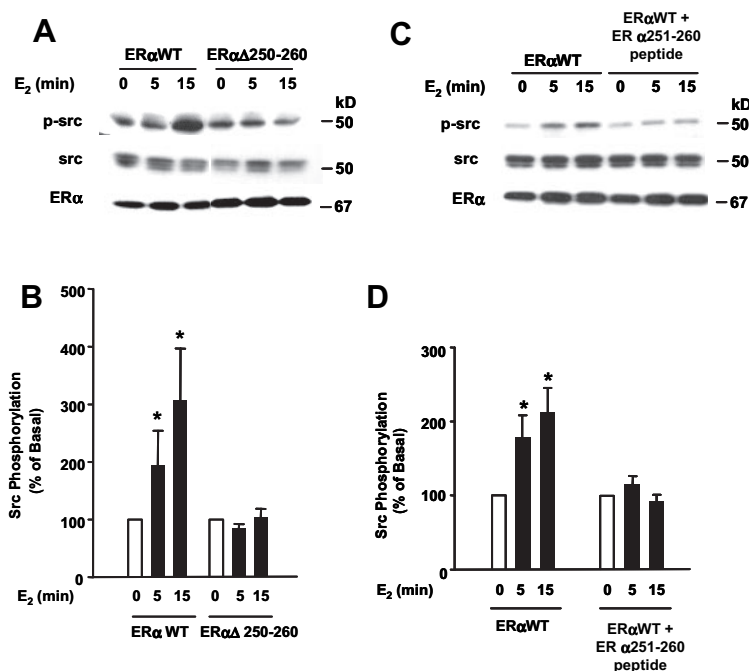
other downstream target of activated G $\beta\gamma$ , erk phosphorylation was also examined (Fig. 6, C and E). Paralleling the findings for src, E<sub>2</sub>-stimulated erk activation was also blunted by  $\beta$ ARK-ct. These cumulative findings show that E<sub>2</sub> activation of ER $\alpha$  releases both G $\alpha$ i and G $\beta\gamma$  without stimulating GTP binding to G $\alpha$ i, and that G $\beta\gamma$  in turn activates the immediate downstream signaling targets src and erk, which are of known importance to multiple nongenomic ER $\alpha$  actions (1–5).

## ER $\alpha$ -G Protein Interactions and E<sub>2</sub> Modulation of Endothelial Cell Function

The importance of direct ER $\alpha$ -G protein interactions to cell function was then addressed in the context of G protein-dependent, plasma membrane-associated ER $\alpha$  activation of eNOS (8). In bovine aortic endothelial cells (BAEC), stimulation of eNOS by E<sub>2</sub> was prevented by ICI 182,780 (Fig. 7A). In further experiments, BAEC were transfected with empty vector or plasmid encoding the ER $\alpha$  mutant ER $\alpha$  $\Delta$ 250–260, which displayed an inability to interact with G $\alpha$ i (Fig. 3E) and an inability to promote E<sub>2</sub> activation of src (Fig. 4, A and B). Of note, in this model system ER $\alpha$  and G $\alpha$ i are present at endogenous levels, and in previous studies we have shown that the overexpression of wild-type ER $\alpha$  in endothelial cells enhances eNOS activation by E<sub>2</sub> (20). Whereas control cells displayed eNOS activation by E<sub>2</sub>, the response was absent in cells expressing ER $\alpha$  $\Delta$ 250–260 (Fig. 7B). In contrast, eNOS activation by vascular endothelial growth factor or acetylcholine was not altered by ER $\alpha$  $\Delta$ 250–260 expression and E<sub>2</sub>-mediated gene transcription assessed using an estrogen response element-luciferase promoter-reporter construct was also not affected (data not shown), indicating that the mutant has a selective dominant-negative action on nongenomic ER function. Similarly, in cells expressing an ER $\alpha$  peptide consisting of amino acids 251–260, E<sub>2</sub> stimulation of eNOS was fully impaired (Fig. 7C) but E<sub>2</sub>-mediated gene transcription was unchanged (data not shown). To test the requirement for ER $\alpha$ -G protein interactions in the modulation of an endothelial cell phenotype of relevance to E<sub>2</sub>-related cardioprotection, the impact of the dominant-negative mutant ER $\alpha$  $\Delta$ 250–260 on E<sub>2</sub>-induced attenuation of monocyte adhesion was evaluated (Fig. 7, D and E). In BAEC transfected with empty vector, the marked increase in monocyte adhesion caused by lipopolysaccharide (LPS) was fully prevented by E<sub>2</sub>. The effect of E<sub>2</sub> was due to nongenomic activation of eNOS because it was abrogated by nitric oxide synthase (NOS) antagonism with *N*-nitro-L-arginine methyl ester and the hormone did not alter eNOS enzyme abundance (Fig. 7E, *inset*). In contrast, in cells expressing the dominant-negative mutant ER $\alpha$  (ER $\alpha$  $\Delta$ 250–260), the E<sub>2</sub>-related, NO-dependent decrease in monocyte adhesion was absent. Thus, the direct interactions between ER $\alpha$  and G proteins are required for E<sub>2</sub>-induced nongenomic actions in endothelial cells of significance to vascular health and disease.

## DISCUSSION

SHRs including ER $\alpha$ , ER $\beta$ , AR, GR, and VDR mediate a variety of nongenomic responses that govern the behaviors of multiple cell types, and evidence has accumulated that in many contexts these processes



**Fig. 4.** Disruption of ER $\alpha$  Interaction with G $\alpha$ i Prevents src Activation by E<sub>2</sub>

A, COS-7 cells were transfected with plasmid encoding WT ER $\alpha$  or ER $\alpha$  $\Delta$ 250–260 and incubated with  $10^{-8}$  M E<sub>2</sub> for 0–15 min, and src activation was evaluated by immunoblot analyses of cell lysates using anti-phospho-src (p-src) polyclonal and anti-src monoclonal antibodies. B, Cumulative results for src phosphorylation expressed as percentage in nontreated cells (basal) for three independent studies (mean  $\pm$  SEM; \*,  $P < 0.05$  vs. basal). C, COS-7 cells were transfected with plasmid encoding WT ER $\alpha$  and either empty vector or a plasmid encoding His-tagged peptide comprised of amino acids 251–260 of ER $\alpha$ , incubated with  $10^{-8}$  M E<sub>2</sub> for 0–15 min, and evaluated for src activation. D, Cumulative results for src phosphorylation for three independent studies (mean  $\pm$  SEM; \*,  $P < 0.05$  vs. basal).

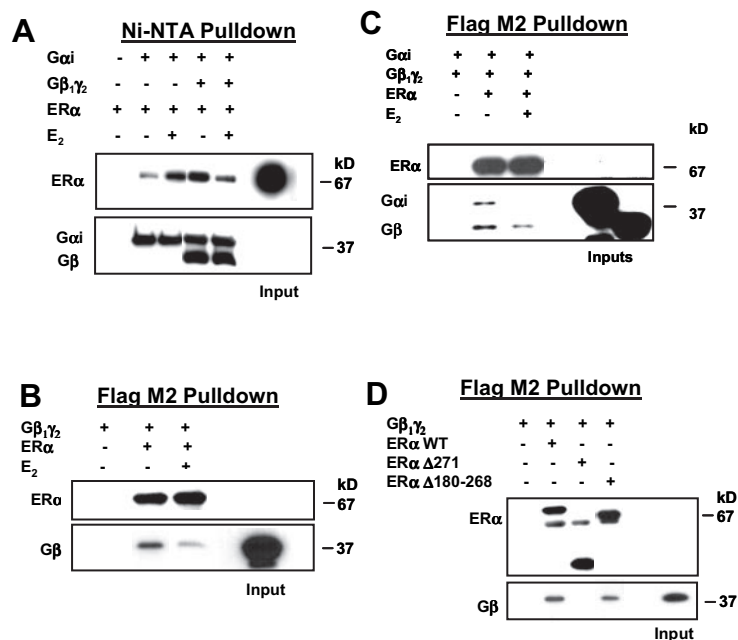
are G protein dependent (16–19, 21, 22). With a focus on ER $\alpha$ , we have demonstrated for the first time direct interactions between an SHR and G proteins, and have determined that such interactions are critically involved in nongenomic steroid hormone signaling.

In pull-down experiments with purified recombinant proteins, we first showed that there is a direct protein-protein interaction between ER $\alpha$  and monomeric G $\alpha$ i, which is enhanced specifically by E<sub>2</sub>. We also demonstrated that the interaction is altered by modifications of G $\alpha$ i that govern its interactions with classical GPCRs (10). Using PTX in studies of src phosphorylation, we further determined that G $\alpha$ i interaction with ER $\alpha$  is an essential proximal process in membrane ER $\alpha$  signaling.

In experiments evaluating whether the interaction observed between ER $\alpha$  and G $\alpha$ i is shared by other SHRs capable of nongenomic signaling involving G proteins (1, 2, 17–19), we found that ER $\beta$  and AR also display direct binding with G $\alpha$ i that is enhanced by their respective steroid hormone ligands. These observations are consistent with the parallel capacity of membrane-associated ER $\alpha$  and ER $\beta$  to promote signaling to eNOS in cultured endothelial cells (21), and the ability of androgens to mediate PTX-sensitive signaling in cell types as diverse as neurons and skeletal muscle (17, 22). In contrast, we observed that GR and

VDR do not bind to G $\alpha$ i. Thus, direct interaction with G $\alpha$ i is a shared feature of ER $\alpha$  and select SHRs that initiate signaling at the plasma membrane. Direct interactions with G $\alpha$ s or G $\alpha$ q may be operative in the nongenomic functions of other SHRs such as GR and VDR. Consistent with the latter possibility, it has been demonstrated that G $\alpha$ q is required for VDR-induced nongenomic signaling during matrix biogenesis by chondrocytes (19).

In further studies of ER $\alpha$ , pull-down experiments performed with G $\alpha$ i and mutant receptor proteins revealed that the region of ER $\alpha$  between amino acids 250 and 260 mediates the direct binding to G $\alpha$ i. In addition, a peptide representing amino acids 251–260 of ER $\alpha$  disrupted the interaction between the wild-type receptor and G $\alpha$ i. Furthermore, the ER $\alpha$  mutant lacking amino acids 250–260 was incapable of activating src in COS-7 cells, and in cells expressing wild-type ER $\alpha$  the coexpression of an HA-tagged peptide representing amino acids 251–260 blocked nongenomic signaling to src. Thus, we have identified amino acids 251–260 of ER $\alpha$  as a G $\alpha$ i binding domain that is critically involved in nongenomic signaling by the receptor. Because there is negligible homology between these amino acids and the corresponding regions of ER $\beta$  and AR, which we show also bind directly to G $\alpha$ i, detailed mutagenesis will now be required to identify



**Fig. 5.** ER $\alpha$  Interacts Directly with G $\beta\gamma$

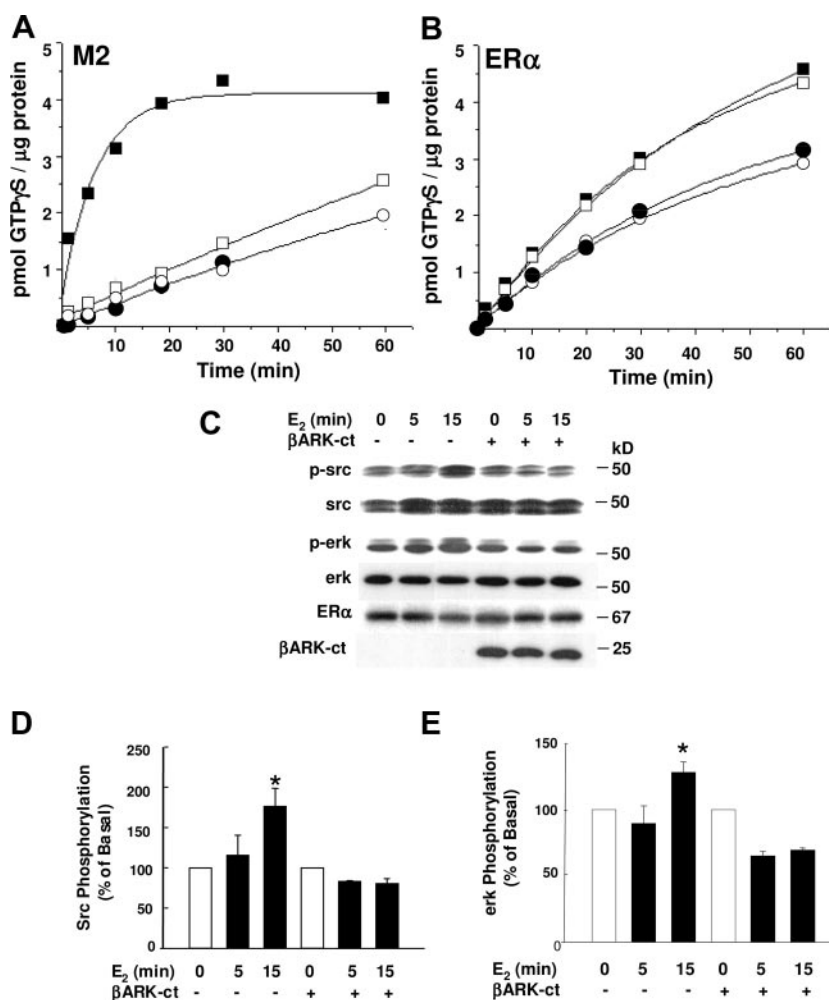
A, Recombinant ER $\alpha$  protein was pulled down with myristoylated His<sub>6</sub>-G $\alpha$ i-GDP using Ni-NTA in the absence or presence of recombinant G $\beta$  $\gamma$ <sub>2</sub> and 10<sup>-8</sup> M E<sub>2</sub>. Immunoblot analyses were performed for ER $\alpha$ , G $\alpha$ i, and G $\beta$ . B, Purified G $\beta$  $\gamma$ <sub>2</sub> was pulled down with recombinant Flag-tagged wild-type ER $\alpha$  in the absence or presence of E<sub>2</sub>. C, Purified G $\alpha$ i-GDP and G $\beta$  $\gamma$ <sub>2</sub> were pulled down using Flag-tagged wild-type ER $\alpha$  bait in the absence or presence of E<sub>2</sub>. D, Comparisons were made in simultaneous G $\beta$  $\gamma$ <sub>2</sub> pull-downs with recombinant Flag-tagged wild-type ER $\alpha$  (WT) vs. ER $\alpha$  $\Delta$ 271–595 vs. ER $\alpha$  $\Delta$ 180–268, with a nonspecific band noted at 50 kDa in all samples in immunoblots for ER $\alpha$ .

the domains within ER $\beta$  and AR mediating this interaction. Interestingly, the G $\alpha$ i binding domain of ER $\alpha$  resides within nuclear localization signal 3 (23), raising the intriguing possibility that there are competitive mechanisms dictating the relative function of the receptor at the plasma membrane and in the nucleus.

Additional pull-down experiments focused on the role of G $\beta\gamma$  in ER $\alpha$ -G $\alpha$ i coupling. We found that G $\beta\gamma$  interacts directly with ER $\alpha$  via a receptor domain(s) residing within amino acids 271–595, which is distinct from the G $\alpha$ i binding domain, that the interaction with G $\beta\gamma$  promotes receptor interaction with G $\alpha$ i, and that the complex formed by ER $\alpha$  and heterotrimeric GDP-bound G $\alpha\beta\gamma$  is disrupted upon E<sub>2</sub> activation of the receptor. We also observed that G $\beta\gamma$  is liberated from ER $\alpha$  by E<sub>2</sub> in the absence of G $\alpha$ i. In studies of the kinetics of G $\alpha$ i nucleotide binding, the activation of the M2 muscarinic receptor serving as a positive control promoted rapid GTP $\gamma$ S binding to G $\alpha$ i, whereas E<sub>2</sub> stimulation of ER $\alpha$  did not. Therefore, G $\beta\gamma$  is released by ER $\alpha$  independently of conventional GTP binding to G $\alpha$ i and the resulting conformational change in G $\alpha$ i that disassociates the  $\beta\gamma$  dimer during signaling by classical GPCRs (10, 11). We postulate that the liberation of G $\beta\gamma$  is mediated alternatively by conformational changes that occur in ER $\alpha$  upon ligand binding. Similar changes in ER $\alpha$  conformation are known to modify the interaction of the receptor with nuclear cofactors (24, 25). Evidence that conformational

changes may impact on ER $\alpha$ -G protein interactions lies in our findings in pull-downs with ICI 182,780, which modify ER $\alpha$  conformation in a manner that is unique compared with E<sub>2</sub> (24, 25). Under certain conditions, ICI 182,780 reversed E<sub>2</sub> effects on ER $\alpha$ -G protein interactions, under other conditions the ICI compound independently altered ER $\alpha$ -G protein interactions, and the modulation of ER $\alpha$ -G protein interactions by both E<sub>2</sub> and ICI 182,780 differed whether or not the G proteins were in heterotrimeric form. Furthermore, experiments in ER $\alpha$ -expressing COS-7 cells showed that cotransfection with  $\beta$ ARK-ct attenuates E<sub>2</sub>-induced srk and erk activation, indicating that the liberated G $\beta\gamma$  modulates downstream signaling. Thus, we have identified a novel means of G protein activation that provides greater diversity of function of an SHR.

The importance of direct ER $\alpha$ -G protein interactions to cell function was addressed in studies of ER $\alpha$  activation of eNOS in cultured endothelium. This process is critically involved in the vascular actions of E<sub>2</sub> that underlie the lower risk of cardiovascular disease in premenopausal women vs. men and the potential of estrogen replacement therapy to be cardioprotective (26). The disruption of ER $\alpha$ -G $\alpha$ i interaction prevented E<sub>2</sub>-induced eNOS activation, which was ER dependent, and it also negated the resulting attenuation of monocyte adhesion that is highly relevant to the initiation of atherosclerosis (26). As such, ER $\alpha$ -G $\alpha$ i interaction plays an important



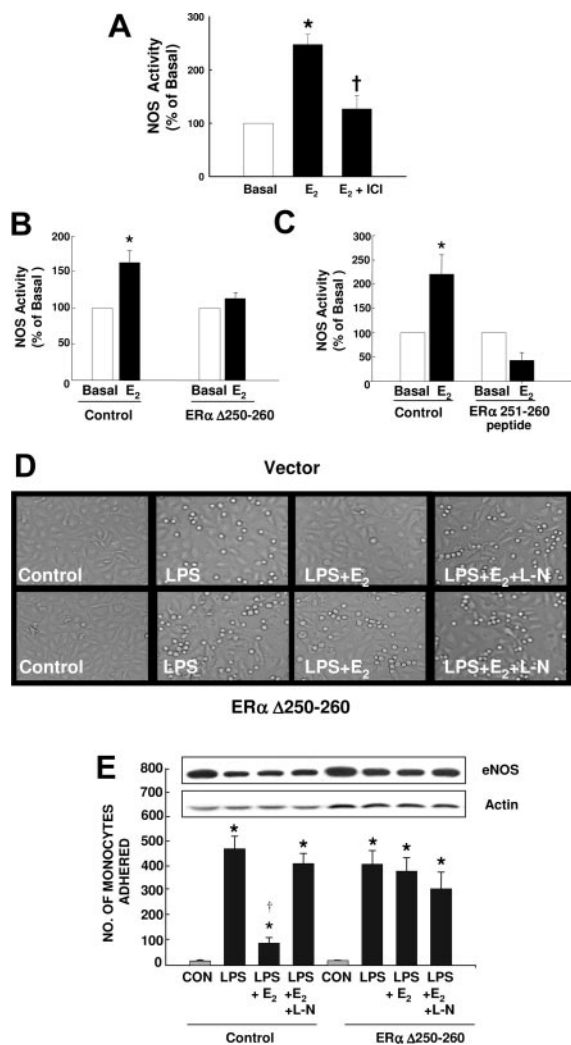
**Fig. 6.** Signal Initiation by E<sub>2</sub> and ER $\alpha$  Complexed with G $\alpha\beta\gamma$  Is Mediated by G $\beta\gamma$  Independent of Guanine Nucleotide Exchange

A and B, The kinetics of GPCR and ER $\alpha$  stimulation of membrane-bound G protein GTP binding differ. A, Membranes from Sf9 cells infected with baculoviruses that express His<sub>6</sub>-tagged-G $\alpha$ i, G $\beta$ , and G $\gamma$ , and either no additional virus or the M2 muscarinic receptor virus were incubated with [<sup>35</sup>S]GTP $\gamma$ S at 30 C in the absence or presence of 10<sup>-6</sup> M carbachol. Aliquots of reaction mixtures were taken at the indicated time points, quenched, and Ni-NTA resin was used to pull down the detergent-extracted G $\alpha$ i. The amount of His-tagged G protein-bound [<sup>35</sup>S]GTP $\gamma$ S was determined by liquid scintillation counting. B, Parallel studies of ER $\alpha$  were performed in the absence or presence of 10<sup>-8</sup> M E<sub>2</sub>. In A and B, results for G $\alpha\beta\gamma$  alone are indicated by *circles* and those for G $\alpha\beta\gamma$  plus receptor by *squares*, and *open* and *closed symbols* represent findings in the absence and presence of ligand, respectively. Values shown in A and B are means for n = 2, and results were confirmed in three separate experiments. C, COS-7 cells transfected with plasmid encoding wild-type ER $\alpha$  and either empty vector or the cDNA for the  $\beta$ ARK-ct were incubated with 10<sup>-8</sup> M E<sub>2</sub> for 0–15 min, and src and erk activation was evaluated by immunoblot analyses of cell lysates using anti-phospho-src (p-src), anti-phospho-erk (p-erk), or anti-src or anti-erk antibodies. Cumulative results are shown for src phosphorylation (D) and erk phosphorylation (E) expressed as percentage in non-E<sub>2</sub>-treated cells (basal) for three independent studies (mean  $\pm$  SEM; \*, P < 0.05 vs. basal).

role in dictating the phenotype of a cell type with well-recognized responses to E<sub>2</sub>.

The mechanisms that we have elucidated in which E<sub>2</sub> initiates downstream nongenomic responses by liberating G $\beta\gamma$  from G $\alpha$ i and ER $\alpha$  upon ligand activation independent of guanine nucleotide exchange add considerably to the processes described to date for nongenomic estrogen signaling. Another example is the potential role of direct estrogen binding to the GPCR GPR30. This event occurs in the endoplasmic reticulum, and it is thought to promote intracellular

calcium mobilization and the generation of PIP3 in the nucleus (27). Additional mechanisms include the involvement of adaptor proteins such as the modulator of nongenomic actions of the ER (MNAR) and striatin, which bind to ER to promote the maintenance of a signaling module (28, 29). In the emerging field of nongenomic endocrinology, our discovery of direct ER $\alpha$ :G protein interactions provides important new understanding of the proximal mechanisms by which proteins classically known to serve as transcription factors exhibit a second fundamental capacity to ini-



**Fig. 7.** Disruption of ER $\alpha$ -G $\alpha$ i Interaction in Endothelial Cells Attenuates ER-Dependent E<sub>2</sub> Activation of eNOS and Resulting Antagonism of Monocyte Adhesion

A, eNOS activation in BAEC was assessed in the presence of buffer alone (basal),  $10^{-8}$  M E<sub>2</sub>, or E<sub>2</sub> plus  $10^{-5}$  M ICI 182,780 for 15 min. B and C, BAEC were transfected with empty vector (control) vs. plasmid encoding ER $\alpha$ Δ250–260 (B), or with empty vector vs. a plasmid that expressed a peptide consisting of amino acids 251–260 of the receptor (ER $\alpha$ 251–260) (C), and E<sub>2</sub>-stimulated eNOS activity was assessed. In A–C, values are mean  $\pm$  SEM; n = 6. \*,  $P < 0.05$  vs. basal; †,  $P < 0.05$  vs. no ICI 182,780. D, Monocyte adhesion was assessed in BAEC transfected with an empty vector (*upper panels*) or a plasmid that expressed ER $\alpha$ Δ250–260 (*lower panels*) and treated with medium alone (control), LPS (100 ng/ml), LPS plus E<sub>2</sub> ( $10^{-8}$  M), or LPS plus E<sub>2</sub> plus nitro-L-arginine methyl ester (L-N) (2 mM). Images are representative optical fields. E, Cumulative findings for monocytes adhered per  $\times 20$  magnification field; mean  $\pm$  SEM; n = 4–5. \*,  $P < 0.05$  vs. control; †,  $P < 0.05$  vs. LPS alone. *Inset* shows immunoblot analyses for eNOS and actin in the various treatment groups.

tiate hormone signaling at the plasma membrane. This work also reveals a new role for G protein signaling outside of conventional GPCR activation. It is anti-

patated that future efforts in this realm will enable us to continue to reveal the intricacies of SHR biology dictating ultimate cellular responses.

## MATERIALS AND METHODS

### Production of Recombinant G $\alpha$ <sub>i1</sub>, G $\beta$ $\gamma$ , ER $\alpha$ , and mutant ER $\alpha$

His-tagged G $\alpha$ <sub>i1</sub> was purified from *Escherichia coli* that had been transformed with a plasmid encoding rat G $\alpha$ <sub>i1</sub> alone or together with a plasmid encoding yeast *N*-myristoyltransferase to produce myristoylated G $\alpha$ <sub>i</sub> (30). G $\beta$ <sub>1</sub> $\gamma$ <sub>2</sub> dimers were synthesized and purified from Sf9 cells as previously described (31). Baculoviruses encoding Flag-tagged wild-type human ER $\alpha$  and mutant ER $\alpha$  truncated proteins were produced and amplified using the Bac-to-Bac Sf9 cell transfection system (Invitrogen, Carlsbad, CA). To create the constructs for Flag-tagged wild-type ER $\alpha$  and the truncation mutants ER $\alpha$ Δ271–595, ER $\alpha$ Δ185–251, ER $\alpha$ Δ261–271, and ER $\alpha$ Δ250–260, the Flag-tag was first inserted N-terminally into the wild-type and the mutant plasmids in pCDNA3.1 using oligonucleotides encoding the heptapeptide tag MDYKDDDK and the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). Using EcoR1 restriction sites, the wild-type and mutant receptor forms with Flag tags were transferred into pFASTBAC1 (Invitrogen) for expression in Sf9 cells. Constructs for Flag-tagged ER $\alpha$ Δ1–175 and ER $\alpha$ Δ185–268 were kindly provided by Dr. W. Lee Kraus (Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY). The sequence of all constructs was verified. To prepare the recombinant proteins, Sf9 cells growing in IPL41 medium were infected with baculovirus for 48 h, pelleted, and homogenized in lysis buffer [20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 20% glycerol, 1 mM dithiothreitol, and protease inhibitor cocktail (Calbiochem, San Diego, CA)], and lysates were subjected to centrifugation at  $12,000 \times g$  at 4 C. Lysates were incubated with Anti-Flag M2 affinity gel (Sigma, St. Louis, MO) at 4 C to isolate the Flag-tagged proteins. After four washes, the proteins were eluted by competition with flag peptide. Purity assessed by SDS-PAGE, and Coomassie blue staining was consistently greater than 95%.

### Protein Interaction Analyses Using Pull-Downs

Purified myristoylated His-tagged G $\alpha$ <sub>i1</sub> (300 nM) was incubated in 500  $\mu$ l of 20 mM HEPES buffer (pH 8.0) containing 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 4% glycerol, 0.05% C12E10, and protease inhibitor cocktail (Calbiochem), with 30  $\mu$ M GDP or GTP $\gamma$ S added for 1 h at 30 C. Purified Flag-tagged ER $\alpha$  proteins were added, plus or minus E<sub>2</sub> at  $10^{-5}$ – $10^{-8}$  molar concentrations, and reactions were incubated at 4 C for 1 h with gentle agitation. Further incubation was performed for 1 h with Ni-nitrilotriacetic acid (NTA) resin (Qiagen, Valencia, CA) to allow binding of His-tagged G $\alpha$ <sub>i1</sub>. Samples were washed with the 20 mM HEPES buffer, and the resin was pelleted and suspended in SDS-PAGE sample buffer. After resolution by 10% SDS-PAGE, immunoblot analyses were performed with the G $\alpha$ <sub>i1/2</sub>-specific antiserum B087 (32), and mouse monoclonal antibodies Ab-15 (Labvision, Fremont, CA) or AER320 (Labvision) directed against ER $\alpha$ . In selected experiments, the impact of ICI 182,780 ( $10^{-5}$  M) was determined. In other studies, a peptide representing amino acids 251–260 of ER $\alpha$  (MKGGIRKDRR) or scrambled peptide (GRGKRIRDKM) was added to the pull-downs ( $10\times$  relative to wild-type ER $\alpha$ ). Additional pull-downs were performed with myristoylated G $\alpha$ <sub>i1</sub>-GDP and recombinant ER $\beta$  (Invitrogen) or recombinant AR in truncated form (amino acids 606–

902) (Invitrogen). Flag pull-down experiments were performed similarly using the Flag-tagged wild-type ER $\alpha$  and mutant ER $\alpha$  proteins, G $\alpha_i$  and/or G $\beta_1\gamma_2$  and Anti-Flag M2 affinity gel (Sigma). In additional experiments, protein interactions were evaluated using COS-7 cell plasma membranes. COS-7 cells were transfected with cDNAs for ER $\beta$ , AR (kindly provided by Dr. Michael McPhaul, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX), GR, or VDR, and 48 h later plasma membranes were isolated as previously described (21). The plasma membranes were then used in pull-down experiments with myristoylated G $\alpha_{i1}$ -GDP done in the absence or presence of  $10^{-5}$  M E $_2$ , dihydrotestosterone, dexamethasone, or 1,25-dihydroxy vitamin D $_3$ , respectively. The Ni-NTA eluted samples were resolved by 10% SDS-PAGE, and immunoblot analyses were performed with receptor-specific antibodies for ER $\beta$  and VDR (Affinity BioReagents, Golden, CO) and AR and GR (Santa Cruz Biotechnology, Santa Cruz, CA), or with the G $\alpha_{i1/2}$  protein-specific antiserum B087.

### Cell Culture and Transfection

COS-7 cells (American Type Culture Collection, Manassas, VA) grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum were transfected with cDNA encoding wild-type human ER $\alpha$  or ER $\alpha\Delta 250-260$  in pCDNA3.1 using LipofectAMINE Plus (Invitrogen). In selected studies, cells were co-transfected with either empty vector or pLP-CMV-HA(ER $\alpha 251-260$ ), an HA-tagged peptide comprised of amino acids 251–260 of ER $\alpha$ , or with empty vector vs. cDNA for  $\beta$ ARK-ct kindly provided by Dr. Robert Lefkowitz (Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC) (33). Primary BAEC were cultured and maintained as previously described and used within seven passages (34). BAEC were transfected with either empty vector vs. ER $\alpha\Delta 250-260$ , or with empty vector vs. pLP-CMV-HA(ER $\alpha 251-260$ ). The sequences of all constructs were verified, and expression was confirmed by immunoblot analyses.

### Src and erk Activation by Immunoblot Analyses

To assess src activation, COS-7 cells were treated with  $10^{-8}$  M E $_2$  for 0–15 min and lysed, and immunoblot analyses were performed using anti-phospho-tyrosine-416 Src polyclonal antibody (Cell Signaling Technology, Danvers, MA) and anti-Src monoclonal antibody (Santa Cruz Biotechnology). To assess erk activation, immunoblotting was performed with anti-phospho-erk polyclonal antibody (Promega, Madison, WI) and anti-erk2 monoclonal antibody (Upstate Biotechnology, Charlottesville, VA).

### GTP $\gamma$ S Binding Studies

Sf9 cells were grown in IPL41 medium and infected with baculoviruses that expressed His $_6$ -G $\alpha_i$ , G $\beta_1$ , and G $\gamma_2$ , and either no additional virus or M2 muscarinic receptor or ER $\alpha$  baculoviruses. Forty-eight hours later, cell membranes were harvested, homogenized into buffer containing 20 mM HEPES, 150 mM NaCl, 2 mM MgSO $_4$ , and 1 mM EDTA (pH 8.0) and used for GTP $\gamma$ S binding time course studies. [ $^{35}$ S]GTP $\gamma$ S was added to the membranes at 30 C in the presence or absence of ligand ( $10^{-6}$  M carbachol or  $10^{-6}$  M E $_2$  or  $10^{-5}$  M ICI 182,780) to initiate the reactions and aliquots were removed at specific time points. Each reaction aliquot was quenched in stop buffer (300 mM MgCl $_2$ , 3.0 mM GDP, 3.0 mM GTP) and extracted with 1% sodium cholate for 1 h at 4 C. After centrifugation at 100,000  $\times$  g for 20 min, the extracts were adsorbed onto Ni-NTA in a buffer containing 20 mM HEPES (pH 8.0), 100 mM NaCl, 1 mM MgCl $_4$ , 10  $\mu$ M GTP, 0.5% C12E10 (Sigma) to pull down His-tagged G $\alpha_i$ -GTP $\gamma$ S. The amount of G $\alpha_i$ -GTP $\gamma$ S (picomoles per microgram of membrane protein) was determined by liquid scintillation counting.

### NOS Activation

NOS activation was assessed in intact BAEC by measuring L-[ $^{14}$ C]arginine conversion to L-[ $^{14}$ C]citrulline using previously reported methods (35). Cells were treated with  $10^{-8}$  M E $_2$  in the absence or presence of  $10^{-5}$  M ICI 182,780. Stimulated activity is expressed as a percentage of basal activity, and results were confirmed in three independent experiments.

### Monocyte Adhesion Assays

The adhesion of monocytes to BAEC was evaluated as previously described (35). Near-confluent BAEC were treated with medium alone or medium plus LPS (100 ng/ml) for 18 h in the absence or presence of  $10^{-8}$  M E $_2$  with or without 2 mM nitro-L-arginine methyl ester. U937 cells ( $1 \times 10^6$  per 35-mm plate) were added to each monolayer under rotating conditions, nonadhering cells were removed by gentle washing with PBS, cells were fixed with 1% paraformaldehyde, and the number of adherent cells was counted per  $\times 20$  magnified field. eNOS and actin abundance was evaluated by immunoblot analyses in additional plates of BAEC treated in an identical manner.

### Statistical Analysis

Comparisons were made between multiple groups by ANOVA with Neuman-Keuls *post hoc* testing. Significance was defined as  $P < 0.05$ .

### Acknowledgments

We thank M. Dixon for preparing this manuscript.

Received August 30, 2006. Accepted March 28, 2007.

Address all correspondence and requests for reprints to: Philip W. Shaul, Department of Pediatrics, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390-9063. E-mail: philip.shaul@utsouthwestern.edu.

This work was supported by National Institutes of Health Grants HD30276 (to P.W.S.) and GM34497 (to Alfred Gilman), by an American Heart Association (Texas Affiliate) Postdoctoral Fellowship (0325033Y to G.G.T.), by the Lowe Foundation (to P.W.S.), and by the Crystal Charity Ball Center for Pediatric Critical Care Research (to P.W.S.).

Disclosure Statement: The authors have nothing to disclose.

### REFERENCES

1. Norman AW, Mizwicki MT, Norman DP 2004 Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. *Nat Rev Drug Discov* 3:27–41
2. Cheskis BJ 2004 Regulation of cell signalling cascades by steroid hormones. *J Cell Biochem* 93:20–27
3. Osborne CK, Schiff R 2005 Estrogen-receptor biology: continuing progress and therapeutic implications. *J Clin Oncol* 23:1616–1622
4. Levin ER 2005 Integration of the extranuclear and nuclear actions of estrogen. *Mol Endocrinol* 19:1951–1959
5. Kelly MJ, Levin ER 2001 Rapid actions of plasma membrane estrogen receptors. *Trends Endocrinol Metab* 12: 152–156
6. Chambliss KL, Shaul PW 2002 Estrogen modulation of endothelial nitric oxide synthase. *Endocr Rev* 23:665–686
7. Garban HJ, Marquez-Garban DC, Pietras RJ, Ignarro LJ 2005 Rapid nitric oxide-mediated S-nitrosylation of es-

- trogen receptor: regulation of estrogen-dependent gene transcription. *Proc Natl Acad Sci USA* 102:2632–2636
8. 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 $\alpha$ (i). *J Biol Chem* 276:27071–27076
  9. Razandi M, Pedram A, Park ST, Levin ER 2003 Proximal events in signaling by plasma membrane estrogen receptors. *J Biol Chem* 278:2701–2712
  10. Cabrera-Vera TM, Vanhauwe J, Thomas TO, Medkova M, Preininger A, Mazzoni MR, Hamm HE 2003 Insights into G protein structure, function, and regulation. *Endocr Rev* 24:765–781
  11. Marinissen MJ, Gutkind JS 2001 G-protein-coupled receptors and signaling networks: emerging paradigms. *Trends Pharmacol Sci* 22:368–376
  12. Kozasa T, Gilman AG 1995 Purification of recombinant G proteins from Sf9 cells by hexahistidine tagging of associated subunits. Characterization of  $\alpha$ 12 and inhibition of adenylyl cyclase by  $\alpha$ 2. *J Biol Chem* 270:1734–1741
  13. Posner BA, Mukhopadhyay S, Tesmer JJ, Gilman AG, Ross EM 1999 Modulation of the affinity and selectivity of RGS protein interaction with G $\alpha$  subunits by a conserved asparagine/serine residue. *Biochemistry (Mosc)* 38:7773–7779
  14. Luttrell LM, Daaka Y, Lefkowitz RJ 1999 Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr Opin Cell Biol* 11:177–183
  15. Parsons SJ, Parsons JT 2004 Src family kinases, key regulators of signal transduction. *Oncogene* 23:7906–7909
  16. Hafezi-Moghadam A, Simoncini T, Yang E, Limbourg FP, Plumier JC, Rebsamen MC, Hsieh CM, Chui DS, Thomas KL, Prorock AJ, Laubach VE, Moskowitz MA, French BA, Ley K, Liao JK 2002 Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. *Nat Med* 8:473–479
  17. Estrada M, Espinosa A, Muller M, Jaimovich E 2003 Testosterone stimulates intracellular calcium release and mitogen-activated protein kinases via a G protein-coupled receptor in skeletal muscle cells. *Endocrinology* 144:3586–3597
  18. Maier C, Runzler D, Schindelar J, Grabner G, Waldhausl W, Kohler G, Luger A 2005 G-protein-coupled glucocorticoid receptors on the pituitary cell membrane. *J Cell Sci* 118:3353–3361
  19. Schwartz Z, Sylva VL, Larsson D, Nemere I, Casasola D, Dean DD, Boyan BD 2002 1 $\alpha$ ,25(OH) $_2$ D $_3$  regulates chondrocyte matrix vesicle protein kinase C (PKC) directly via G-protein-dependent mechanisms and indirectly via incorporation of PKC during matrix vesicle biogenesis. *J Biol Chem* 277:11828–11837
  20. Chen Z, Yuhanna IS, Galcheva-Gargova Z, Karas RH, Mendelsohn ME, Shaul PW 1999 Estrogen receptor  $\alpha$  mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. *J Clin Invest* 103:401–406
  21. Chambliss KL, Yuhanna IS, Anderson RG, Mendelsohn ME, Shaul PW 2002 ER $\beta$  has nongenomic action in caveolae. *Mol Endocrinol* 16:938–946
  22. Shakil T, Hoque AN, Husain M, Belsham DD 2002 Differential regulation of gonadotropin-releasing hormone secretion and gene expression by androgen: membrane versus nuclear receptor activation. *Mol Endocrinol* 16:2592–2602
  23. Ylikomi T, Bocquel MT, Berry M, Gronemeyer H, Chambon P 1992 Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. *EMBO J* 11:3681–3694
  24. Paige LA, Christensen DJ, Gron H, Norris JD, Gottlin EB, Padilla KM, Chang CY, Ballas LM, Hamilton PT, McDonnell DP, Fowlkes DM 1999 Estrogen receptor (ER) modulators each induce distinct conformational changes in ER $\alpha$  and ER $\beta$ . *Proc Natl Acad Sci USA* 96:3999–4004
  25. McDonnell DP 2004 The molecular determinants of estrogen receptor pharmacology. *Maturitas* 48(Suppl 1):S7–S12
  26. Mendelsohn ME, Karas RH 2005 Molecular and cellular basis of cardiovascular gender differences. *Science* 308:1583–1587
  27. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER 2005 A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* 307:1625–1630
  28. Wong CW, 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
  29. Lu Q, Pallas DC, Surks HK, Baur WE, Mendelsohn ME, Karas RH 2004 Striatin assembles a membrane signaling complex necessary for rapid, nongenomic activation of endothelial NO synthase by estrogen receptor  $\alpha$ . *Proc Natl Acad Sci USA* 101:17126–17131
  30. Mumby SM, Linder ME 1994 Myristoylation of G-protein  $\alpha$  subunits. *Methods Enzymol* 237:254–26831
  31. Kozasa T 1999 Purification of recombinant G protein  $\alpha$  and  $\beta\gamma$  subunits from Sf9 cells. In: Manning DR, ed. *G proteins: techniques of analysis*. Boca Raton, FL: CRC Press; 23–38.
  32. Linder ME, Middleton P, Hepler JR, Taussig R, Gilman AG, Mumby SM 1993 Lipid modifications of G proteins:  $\alpha$  subunits are palmitoylated. *Proc Natl Acad Sci USA* 90:3675–3679
  33. Koch WJ, Hawes BE, Inglese J, Luttrell LM, Lefkowitz RJ 1994 Cellular expression of the carboxyl terminus of a G protein-coupled receptor kinase attenuates G $\beta\gamma$ -mediated signaling. *J Biol Chem* 269:6193–6197
  34. Seetharam D, Mineo C, Gormley AK, Gibson LL, Vongpatanasin W, Chambliss KL, Hahner LD, Cummings ML, Kitchens RL, Marcel YL, Rader DJ, Shaul PW 2006 High-density lipoprotein promotes endothelial cell migration and reendothelialization via scavenger receptor-B type I. *Circ Res* 98:63–72
  35. Mineo C, Gormley AK, Yuhanna IS, Osborne-Lawrence S, Gibson LL, Hahner L, Shohet RV, Black S, Salmon JE, Samols D, Karp DR, Thomas GD, Shaul PW 2005 Fc $\gamma$ RIIB mediates C-reactive protein inhibition of endothelial NO synthase. *Circ Res* 97:1124–1131

