Forskolin Stimulates Estrogen Receptor (ER) α Transcriptional Activity and Protects ER from Degradation by Distinct Mechanisms

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Estradiol action is mediated by estrogen receptors (ERs), α and β. Estradiol binding initiates ER-mediated transcription and ER degradation, the latter of which occurs via the ubiquitin-proteasome pathway. Inhibition of proteasome activity prevents estradiol-induced ERα degradation and transactivation. In ER-positive GH3 cells (a rat pituitary prolactinoma cell line), forskolin, acting via protein kinase A (PKA), stimulates ERα transcriptional activity without causing degradation, and proteasome inhibition does not block forskolin-stimulated transactivation. Forskolin also protects liganded ERα from degradation. In the current study, we first examined ERα and ERβ transcriptional activity in ER-negative HT22 cells and found that forskolin stimulated ERα-, but not ERβ-dependent transcription, through the ligand-binding domain (LBD). We also identified four mutations (L396R, D431Y, Y542F, and K534E/M548V) on the ERα LBD that selectively obliterated the response to forskolin. In GH3 cells, transfected ERα mutants and ERβ were protected from degradation by forskolin. Ubiquitination of ERα and ERβ was increased by forskolin or estradiol. ERα ubiquitination was diminished by a mutated ubiquitin (K48R) that prevents elongation of polyubiquitin chains for targeting the proteasome. Increased ERα ubiquitination was not affected by the deletion of the A/B domain but significantly diminished in the F domain deletion mutant. Our results indicate distinct and novel mechanisms for forskolin stimulation of ERα transcriptional activity and protection from ligand-induced degradation. It also suggests a unique mechanism by which forskolin increases unliganded and liganded ERα and ERβ ubiquitination but uncouples them from proteasome-mediated degradation regardless of their transcriptional responses to forskolin.

1. Introduction

Estrogens exert their actions through estrogen receptors (ERs), ERα and ERβ, to regulate a variety of physiological functions of the cardiovascular, endocrine, musculoskeletal, nervous, and reproductive systems [1, 2]. ERs are members of the steroid/nuclear receptor superfamily with four major functional domains, including the amino-terminal, ligand-independent transactivation domain (activation function-1, AF-1), the central DNA-binding domain (DBD), the hinge region, and the carboxyl-terminal ligand-binding domain (LBD)/ligand-dependent transactivation (activation function-2, AF-2) [1, 2]. In the classical pathway, ERs undergo a conformational change once bound by estrogens (activation), forming a dimer and then binding to estrogen response elements (EREs) in the transcriptional regulatory regions of target genes [1–3]. Acting as bridging proteins, liganded ERs dynamically recruit transcriptional coactivators and components of the RNA polymerase II transcription initiation complex to enhance target gene transcription [3–5]. Concomitant with increased ERα transcriptional activity, ligand binding also causes ERα protein degradation [6–10]. Deletion of the LBD of human ERα or mutations within this domain that prevent ligand
binding (G521R) and/or the activation of helix 12 for coactivator interaction (D538A, L539A/L540A, D538A/E542A/D545A) decrease ligand-induced proteolysis [6, 9, 11, 12]. These data suggest that specific conformational changes in liganded ERα LBD are important not only for transcriptional activity but also for receptor degradation.

Estradiol-induced ERα protein degradation is mediated by the ubiquitin-proteasome pathway [6, 8–10]. In this pathway, the target proteins are first covalently conjugated with ubiquitin on the lysine residues by three classes of enzymes, including ubiquitin-activating enzymes (Uba), ubiquitin-conjugating enzymes (Ubc), and ubiquitin ligases [13, 14]. Free ubiquitin is then added to the lysine 48 (K48) on the ubiquitin conjugated to target proteins, and this process is repeated to form a polyubiquitination chain on the substrate protein, which is implicated in targeting the proteins to the proteasome for degradation [15–18]. Several studies have shown that ERα becomes ubiquitinated in the presence of estradiol or selective ER modulators (SERMs) [9, 19].

Multiple lines of evidence indicate a functional linkage between ligand-dependent ER transcription and the ubiquitin-proteasome system. Prevention of ERα degradation by proteasome inhibitors, such as MG132 and lactacystin, disrupts estradiol-induced ERα transactivation in HeLa cervical cancer cells, MCF7 breast cancer cells, and GH3 pituitary cells [6, 8, 20]. A temperature-sensitive mutation of the Uba, disrupting protein ubiquitination, abolishes ligand-induced ERα degradation and ERα-mediated transcription [6]. Chromatin immunoprecipitation of estradiol-sensitive gene promoters demonstrates that cyclical occupancy of ERα by liganded ERα is regulated by the proteasome and required for the transcriptional responsiveness of ERα to estradiol [20–22]. Additionally, the ubiquitin ligase E6-associated protein (E6-AP) and suppressor of gal 1 (SUG1/TRIP1), an ATPase subunit of the 26S proteasome complex, are both reported to act as transcription cofactors of ERs [23–25]. Thus, the ligand-dependent transactivation of ERα is tightly linked to its degradation through the ubiquitin-proteasome pathway, but it is unclear if similar changes in conformation and the activation-degradation linkage also hold true for ERβ.

Ligand-independent activation of ER has been described in several cell types, including the uterus, cervix, and pituitary, and there are clearly both context- and signaling-pathway-dependent contributions [26–28]. For example, mitogen-activated protein kinase (MAPK) stimulation of ERα activity occurs through the AF-1 region and potentially via direct phosphorylation of the receptor and coactivators [27, 29–31], whereas either GnRH acting via protein kinase C (PKC)/MAPK pathways [32] or cAMP acting via protein kinase A (PKA) stimulated transcriptional activity of pituitary ERα [7, 8]. Much less is known about the mechanisms by which these ligand-independent pathways function in ER activation and turnover, and it is unclear whether direct posttranslational modification and/or conformational changes of ER are involved.

We have previously shown that forskolin, which activates adenyl cyclase and increases intracellular levels of cAMP, stimulates ERα-mediated transcriptional activity in rat lactotroph GH3 cells through PKA without causing ERα degradation and that inhibition of proteasome activity had no effect on forskolin-stimulated transcription [8]. The dissociation of forskolin-stimulated, ERα-mediated transcription from both receptor degradation and the requirement for proteasome activity suggests important differences between mechanisms of transactivation by forskolin and estradiol. Forskolin also protects liganded ERα from degradation, suggesting that PKA-dependent pathways may generally stabilize ERα. In this study, we examined the hypothesis that forskolin-induced rat pituitary ERα activation and protection of rat ERα from degradation occurred through separate mechanisms by dissecting the molecular events involved in receptor activation and degradation. We found that only ERα, not ERβ, was stimulated by forskolin, and several mutations in the LBD selectively impacted ERα transcriptional activation by forskolin. However, forskolin treatment protected both ERβ and the transcriptionally inactive mutated ERα from degradation after ligand binding, demonstrating that these two molecular processes can be uncoupled.

2. Materials and Methods

2.1. Chemicals and Antibodies. Cycloheximide and 17β-estradiol were purchased from Sigma (St. Louis, MO), and forskolin was purchased from Tocris (Ellisville, MO). Tumor necrosis factor α (TNFα) and ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) inhibitor as well as lactacystin and MG132 were purchased from Calbiochem (San Diego, CA) and BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA), respectively. Hemagglutinin (HA) and His6 antibodies were purchased from the Hybridoma Core, University of Virginia (Charlottesville, VA) and Novagen (Madison, WI), respectively. The antibody against amino acids 586–600 of the rat ERα (C1355), generated by our lab, was characterized previously [33].

2.2. Plasmids. Rat ERα and ERβ cDNAs were subcloned into the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA) as described previously [34, 35]. To generate HA-tagged ERα, ERα and ERβ cDNAs were excised from the pcDNA3.1 vector with BamHI and EcoRI and then ligated into the pKH3 expression vector, which contained three copies of the HA tag immediately 5′ to the multiple cloning site (graciously provided by Dr. Ian Macara, Vanderbilt University, Nashville, TN) [36]. The pcDNA3.1 and pKH3 vectors both carried the transgene under the control of a cytomegalovirus (CMV) promoter. Site-directed mutagenesis was used to introduce specific amino acid substitution mutations to ERα DBD and LBD using the QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). A list of the mutations is selected because the same mutations on the equivalent residues of human ERα have been reported to (1) interfere PKA phosphorylation (S241E and S253A) [37, 38], (2) alter transcriptional activity in response to estradiol or SERMs (D356V, K367A, L377R, V381R, E385Q, D431Y, D432N, N433K, and D435N).
Y531A, K534E, Y542E, Y542F, and Y542S) [39–41], or (3) perturb LBD helical structures for ligand binding and/or cofactor interaction (N353A, L396R, L408A, N460A, D478A, H493A, and M548V) (in consultation with Dr. Fraydoon Rastinejad). Besides, the double mutant of K534E/M548V was created unexpectedly when cloning rat ERα cDNA to an expression vector, and these substitution mutations were confirmed by DNA sequencing.

In addition, we also used a series of domain deletion mutants derived from the HA-ERα construct to examine the contribution of the individual regions towards ER transcriptional activity and stability. To generate HA-ERα deletion mutants lacking the E/F (ΔE/F), helix 12 and F (ΔH12/F), or F (ΔF) domains, a TGA stop codon was introduced immediately before each of those domains. The HA-ERα A/B deletion (ΔA/B) was constructed by introducing a BamHI restriction enzyme site at the end of the B region of ERα cDNA, followed by excising and subcloning the modified cDNA into the pKH3 vector with BamHI and EcoRI. The HA-tagged ubiquitin vector, containing eight copies of ubiquitin, was kindly provided by Dr. Deborah Lannigan (Vanderbilt University) [42, 43]. His6- and HA-tagged ubiquitin vectors, including both wild-type and K48R, were provided by Drs. Ron Kopito (Stanford University, Stanford CA) and Ze’ev Ronai (Burnham Institute for Medical Research, La Jolla, CA), respectively [44, 45]. The pG3L-2ERE reporter containing two ERE consensus sequences followed by a prolactin TATA box (Promega) was used to analyze the transcriptional activity of ERα [46].

2.3. Luciferase Reporter Assays for Measuring ER-Mediated Transcription. Mouse hippocampal HT22 cells, lacking endogenous ERα or ERβ, were maintained in Cellgro® Dulbecco’s modified Eagle’s medium (DMEM; Mediatech/Fisher, Herndon, VA) containing 10% fetal bovine serum (Gibco/Invitrogen, Grand Island, NY) and 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco/Invitrogen). Cells were plated in phenol red-free DMEM with 5% stripped newborn calf serum (sNCS) at the density of 1 × 10^5 cells per ml in 18 mm well plates. Cells were transiently transfected with the pGL3-2ERE reporter (500 ng/well) plus a control or ER expression vector (10 ng/well) for 18–22 h with FuGENE 6 (Roche, Indianapolis, IN) [8]. Cells were then treated with vehicle, 10 nM estradiol, or 1 µM forskolin for 24 h, then washed with phosphate-buffered saline, and then collected in 200 µl of 1 × Cell Culture Lysis Reagent (Promega, Madison, WI) and later assayed for luciferase activity using a Turner TD-20e luminometer (Sunnyvale, CA). Total protein levels of individual lysates were also determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). Luciferase activity from each sample was normalized to total lysate protein levels as described [8]. Each treatment was performed in triplicate, and experiments were repeated at least three times.

2.4. Immunoblotting for Measuring Levels of Endogenous and Transfected ERs. GH3 cells, a rat pituitary-derived cell line, were plated in DMEM with 5% sNCS at 1.2 × 10^6 cells per 35 mm well. In some studies, cells were transfected with control or HA-tagged ER expression vectors (400 ng/well) with Lipofectamine™ 2000 (Invitrogen) for 18–20 h. Cells were pretreated with vehicle or cycloheximide (20 µg/ml) for 30 min, followed by vehicle, 10 nM estradiol, 1 µM forskolin, or both for 6h. Cells were then collected in gel loading buffer as previously described [8, 46]. Total protein levels of the lysates were determined using BCA Protein Assay (Pierce Chemical Co., Rockford, IL). Individual lysates (~30 µg each) were separated on 8% polyacrylamide-SDS gels and transferred to nitrocellulose membranes (Bio-Rad). Endogenous ERα was detected with ERα antibody, C1355 (1:7,500), while transfected HA-tagged ERα was detected with HA antibody at a concentration of 1:10,000. After rinsing, blots were then incubated in a horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (1:10,000; Amersham Pharmacia Biotech, Arlington Heights, IL) or goat anti-mouse IgG secondary antibody (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h, followed by incubation in SuperSignal® West Pico Chemiluminescence (Pierce Chemical Co.) and detection on Kodak X-OMAT X-ray film (Kodak Co., Rochester, NY). The same blots were reprobed with the β-actin antibody at 1:50,000 (Sigma), then incubated in an HRP-conjugated goat anti-mouse IgG secondary antibody (1:50,000; Jackson ImmunoResearch Laboratories) for 1 h and chemiluminescent detection. With a densitometer, the intensities of ER and β-actin bands on each film were measured and analyzed using ImageQuant (Molecular Dynamics, Inc., Sunnyvale, CA). Relative ER protein level of each sample was calculated by normalizing the intensity of ER to that of β-actin and expressed as a percentage of vehicle-treated controls (as 100%). Each experiment was performed in duplicate wells and repeated at least three times.

2.5. Immunoprecipitation and Immunoblotting for Detecting ER Ubiquitination. GH3 cells plated at a density of 8 × 10^6 cells per 100-mm Corning® cell culture dish (Fisher) were transfected with the expression vectors for HA- or His6-tagged ubiquitin (4 µg/dish) for 18–20 h. Cells were pre-treated with vehicle or MG132 (40 µM) for 1 h, followed by treatment of vehicle, estradiol, forskolin, or both for 6 h. Cells were then collected in M-PER® Mammalian Protein Extraction Reagent (Pierce Chemical Co.) containing N-ethylmaleimide (Sigma) and a cocktail of protease inhibitors. Total lysate protein levels were similarly determined with BCA Protein Assay (Pierce Chemical Co.). Lysates of 500 µl (1 µg/µl) were incubated with the antibodies for ERα (1:250) at 4°C for 18 h, or the antibodies against HA or His6 tags (1:100) at 4°C for 18 h followed by Protein G PLUS-agarose (40 µl; Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4°C for 1 h. Lysates were centrifuged, and the pellets were washed twice with RIPA buffer, followed by separated on 8% polyacrylamide-SDS gels, transferred to nitrocellulose membranes, and subjected to immunoblotting for HA or His6.

2.6. Statistical Analyses. The data of luciferase activities and ER protein levels were statistically analyzed by one-way or...
two-way analysis of variance (ANOVA) to reveal the effects of treatments and ER mutations. A confidence level of \( P < 0.05 \) was considered significant. If there was a significant main effect or interaction, Tukey’s wholly significant difference (WSD) post hoc test was further used for multiple pairwise comparisons.

3. Results

3.1. Forskolin Increases Transcriptional Activity of ERα, but Not ERβ. Which Is Independent of Proteasome Activity. Figure 1(a) depicts the protein domains and sequence similarity of rat ERs and ERβ, which are composed of 600 and 549 amino acids. Between the two ER subtypes, the amino-terminal A/B domain shares a 26% amino-acid identity, while the central C region is near-identical (97%), the hinge, or D, domain shows 23% identity, whereas in the carboxyl terminal, the E and F domains share 59% and 17% amino-acid homology, respectively.

We have previously used GH3 cell line as a model to demonstrate ER-mediated transactivation in response to forskolin, and inhibition of 26S proteasome activity does not disrupt the effect of forskolin on ER transcriptional activity [8]. GH3 cells express both ERα and ERβ although the former seems to be the major subtype [8, 47, 48]. Thus, we first investigated the ER subtype specificity of forskolin-stimulated transactivation using ER-negative HT22 cells transiently transfected with a pGL3-2ERE-luciferase reporter plus either a control, rat ERα, or rat ERβ expression vector. There was no stimulation of theERE-reporter activity by estradiol (1.20 ± 0.18 fold) or forskolin (1.12 ± 0.14 fold) in the absence of ER (Control; Figure 1(b), left panel). Cells transfected with ERα showed increased basal transcriptional activity (4.77 ± 1.01 fold) as well as both estradiol- and forskolin-stimulated ER transcriptional activity (17.01 ± 3.82 and 15.40 ± 3.48 folds, respectively; \( P < 0.05 \)) (Figure 1(b), right panel). In ERβ-transfected cells, estradiol was able to stimulate the ERE-reporter activity (4.92 ± 0.97 fold) (\( P < 0.05 \)), not forskolin (1.7 ± 0.23 fold) (Figure 1(b), middle panel). Our data demonstrate that forskolin stimulation of rat ER transcriptional activity is ER subtype-specific.

Since inhibition of 26S proteasome activity by lactacystin disrupted ligand-dependent, not ligand-independent, transcriptional activity of endogenous ERs in GH3 cells [8], we then examined if estradiol and forskolin stimulation of transfected ERα and ERβ similarly required the 26S-proteasome pathway. HT22 cells, transfected with an ERE-luciferase reporter plus either an ERα or ERβ expression vector, were pretreated with lactacystin (10 \( \mu M \)) for 1h, followed by treatment of estradiol or forskolin for 24h and then measurement of ERE reporter activity. In agreement with Figure 1(b), estradiol stimulated transcriptional activity of ERα (4.10 ± 0.13 fold) and ERβ (3.67 ± 0.54 fold) (\( P < 0.05 \)), which was suppressed by lactacystin at the concentration that had shown to prevent estradiol-induced ERα degradation in GH3 cells (Figure 1(c), gray bars). On the other hand, forskolin increased transcriptional activation of ERs (5.06 ± 0.03 fold), not ERβ (1.71 ± 0.35 fold), and unlike liganded ERα, the forskolin-stimulated ERα transcriptional activity was not affected by lactacystin (5.07 ± 0.31 fold) (\( P < 0.05 \)) (Figure 1(c), closed bars). Our data demonstrate that estradiol stimulation of ERα- and ERβ-mediated transactivation requires the proteasome pathway and that the difference in proteasome participation in transcriptional activity indicates distinct mechanisms underlying the ligand-dependent and ligand-independent activation of ERα.

3.2. Forskolin Increases Transcriptional Activity of ERα through the LBD. After showing that the forskolin stimulation of ER transcriptional activity is ERα-specific, we then identified which ERα region mediated forskolin stimulation. HT22 cells were similarly transfected with an ERE reporter plus an amino-terminal HA-tagged ERα expression vector, including full-length and deletion mutants (Figure 2(a)). One amino- and three carboxyl-terminal deletion mutants were used: ΔA/B, which consisted of the C, D, E, and F domains; ΔE/F, which consisted of the A/B, C, and D domains; ΔH12/F, which lacked helix 12 of the LBD and the F domain; ΔF, in which the F domain was deleted. ΔA/B has ligand-binding activity and retains AF-2 transactivation function whereas ΔE/F retains AF-1.

Lack of the A/B region (ΔA/B panel) did not affect estradiol (6.15 ± 0.66 fold) and forskolin (7.21 ± 0.72 fold) stimulation of ERα transactivation (\( P < 0.05 \)), whereas deletion of the E/F (ΔE/F panel) regions completely eliminated both estradiol- and forskolin-dependent ERα transcriptional activity (1.07 ± 0.07 and 2.14 ± 0.5 folds, respectively) (Figure 2(b)). Deletion of the F region (lacking amino acids 557–600; ΔF panel) reduced but did not abolish ERα transactivation in response to estradiol (3.07 ± 0.66 fold) or forskolin (3.92 ± 0.13 fold) while deletion of both the F region and helix 12 (lacking amino acids 539–600; ΔH12/F panel) completely eliminated the responses to estradiol (1.57 ± 0.03 fold) or forskolin (1.55 ± 0.12 fold) (Figure 2(b)). These data indicate that forskolin-stimulated ERα transcriptional activity requires the LBD, including the helix 12, and the AF-2 seems to be important for forskolin action on transcriptional activity of unliganded ERα.

3.3. Mutation of Specific ERα LBD Residues Selectively Disrupts Forskolin-Stimulated Transactivation. To further dissect how the LBD mediated forskolin-induced ERα activation, we introduced a variety of single or double-residue mutations into the ERα DBD and LBD and tested their effects on the estradiol and forskolin responses (Table 1). Figure 3 shows the sequence of ERα LBD with secondary structure (helixes, coil, and sheet) indicated. Among these mutations, S241E in the DBD mimics phosphorylation at this PKA phosphorylation site homologousto S518 in human ERα. Secondary structure (helixes, coil, and sheet) indicated. Among these mutations, S241E in the DBD mimics phosphorylation at this PKA phosphorylation site homologousto S518 in human ERα. Another group of ERα mutants, including D356V, Y542E, Y542F, and Y542S, were selected because their equivalent residues in human or mouse ERα demonstrated altered transcriptional activity in response to estradiol or SERMS [39, 40]. The remaining ERα mutants, N353A,
L408A, N460A, D478A, and H493A, were created to perturb individual helical structures of the ERα LBD by reducing the size of the amino-acid side chains.

Approximately 50% of the ERα mutants tested, including those in alpha helices (N353A, D356V, K367A, L377R, V381R, N460A, D478A, H493A, K534E, and M548V), β sheets (L408A), potential phosphorylation sites for PKA (S523A), or tyrosine kinases (Y531A), had similar or better transcriptional activity in response to estradiol and forskolin compared to wild-type ERα (Table 1, Group I). Four ERα mutants, S241E (mimicking potential PKA phosphorylation), E385Q, Y542E, and Y542S (around the coactivator interaction), E385Q, Y542E, and Y542S (around the coactivator interaction), were transiently transfected with pGL3-2ERE luciferase reporter (500 ng) plus an expressing vector carrying wild-type or mutated ERα with deletion of A/B (ΔA/B), E/F (ΔE/F), helix12/F (ΔH12/F), and F (ΔF) (10 ng) and treated either vehicle (V), estradiol (E, 10 nM), or forskolin (F, 1 μM) for 24 h. Luciferase activity (mean ± SEM) was normalized and expressed as fold stimulation over the vehicle-treated controls (as 1 fold) from at least three independent experiments performed in triplicate. * P < 0.05 vs. vehicle-treated groups with the same ER expression vector.

Figure 1: Forskolin increases ERα-, but not ERβ-mediated transcription, and forskolin stimulation of ERα transactivation does not require the proteasome pathway. (a) The panel shows the domain organization and sequence homology of rat ERα and ERβ. (b) HT22 cells were transiently transfected with pGL3-2ERE luciferase reporter (500 ng) plus a control (pcDNA3.1), rat ERα, or ERβ expression vector (10 ng). These cells received the treatment of either vehicle (V), estradiol (E, 10 nM), or forskolin (F, 1 μM) for 24 h. Luciferase activity (mean ± SEM) was normalized and expressed as fold stimulation over the vehicle-treated controls (as 1 fold) from at least three independent experiments with triplicate samples. * P < 0.05 vs. vehicle-treated groups (V) with the same ER expression vector. *, P < 0.05 vs. vehicle-treated group with the empty expression vector (Control-V). (c) HT22 cells, transfected with pGL3-2ERE luciferase reporter plasmid (500 ng) plus a rat ERα or ERβ expression vector (10 ng), were pretreated with either vehicle or lactacystin (+Lac, 1 μM), followed by vehicle (V), estradiol (E), or forskolin (F). Normalized luciferase data were expressed as the fold stimulation over vehicle-treated cells carrying the same ER construct (without lactacystin) (1 fold) and expressed as the mean ± SEM from three independent experiments performed in triplicate. *, P < 0.05 vs. vehicle-treated groups (V) with the same ER expression vector (without lactacystin).
ERα Degradation, but Not Forskolin Protection from 3.4. Deletion of the ERα F Domain Inhibits Estradiol-Induced ERα Degradation, but Not Forskolin Protection from Degradation. We have previously shown that endogenous ERα in GH3 cells is not degraded by forskolin and is even protected by forskolin against ligand-dependent degradation [8]. To identify which regions of ERα were required for the action of forskolin on degradation, GH3 cells were transiently transfected with HA-tagged ERα constructs with deletion of the A/B (ΔA/B), E/F (ΔE/F), helix 12/F (ΔH12/F), or F (ΔF). The transfected ERα proteins were distinguished from endogenous ERα by their HA tag. As a representative blot shown in Figure 4(a), both endogenous ERα and transfected ΔA/B protein levels were decreased by estradiol (Lane 2) but protected from degradation by forskolin (Lanes 3 and 4). To discriminate the effects of proteolysis and translation on protein levels, cells were pretreated with cycloheximide to inhibit new protein synthesis. With the presence of cycloheximide, vehicle-treated GH3 cells showed decreased levels of endogenous ERα (V, 81.0 ± 5.2%) and transfected ΔA/B (V, 47.6 ± 4.7%) compared to untreated cells (100%; dash line) (Figure 4(a) and Figure 4(b), left panels), indicating that the truncated ERα seemed to have higher basal turnover rates than the full-length receptor. Estradiol further reduced levels of ERα (E, 59.7 ± 3.9%) and ΔA/B (E, 33.6 ± 3.0%) (P < 0.05). Forskolin not only caused no change in the levels of unliganded ERα (Groups F: ERα = 90.5 ± 7.2% and ΔA/B = 62.5 ± 5.5%) but also protected them from estradiol-stimulated degradation (Groups B: ERα = 78.3 ± 8.3% and ΔA/B = 58.9 ± 5.3%) (Figure 4(b), left panels). In turn, ERα mutant with a deletion

<table>
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<tr>
<th>Residue</th>
<th>Helixb</th>
<th>Vehicle</th>
<th>Estradiol</th>
<th>Forskolin</th>
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<tbody>
<tr>
<td>Wild-type ERα</td>
<td>1.00 ± 0.03</td>
<td>3.60 ± 0.31</td>
<td>4.19 ± 0.35</td>
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| Group 1: No change/increase of estradiol or forskolin stimulation: |
|----------------|----------------|
| N353A | 3 | 1.00 ± 0.07 | 6.76 ± 1.94 | 9.69 ± 2.40 |
| D356V | 3 | 1.00 ± 0.09 | 9.51 ± 0.58 | 11.32 ± 0.47 |
| K367A | 3 | 1.00 ± 0.02 | 2.31 ± 0.05 | 3.07 ± 0.36 |
| L377R | 5 | 1.00 ± 0.03 | 8.17 ± 2.61 | 6.32 ± 0.67 |
| V381R | 5 | 1.00 ± 0.02 | 2.79 ± 0.71 | 2.27 ± 0.16 |
| L408A | S1/S2 | 1.00 ± 0.02 | 3.96 ± 0.56 | 5.25 ± 1.11 |
| N460A | 1.00 ± 0.04 | 7.01 ± 2.07 | 12.16 ± 4.26 |
| D478A | 1.00 ± 0.04 | 6.43 ± 1.82 | 9.43 ± 3.01 |
| H493A | 10 | 1.00 ± 0.05 | 8.93 ± 0.61 | 12.94 ± 4.11 |
| S523A | 11 | 1.00 ± 0.06 | 3.01 ± 0.64 | 4.16 ± 0.64 |
| Y531A | 11 | 1.00 ± 0.03 | 11.10 ± 1.90 | 15.10 ± 5.01 |
| K534E | 11 | 1.00 ± 0.05 | 13.98 ± 1.51 | 5.61 ± 0.54 |
| M548V | 12 | 1.00 ± 0.12 | 13.83 ± 1.82 | 11.45 ± 2.00 |

| Group II: Loss of both estradiol and forskolin stimulation: |
|----------------|----------------|
| S241E | DBD | 1.00 ± 0.10 | 1.79 ± 0.41 | 1.24 ± 0.12 |
| E385Q | 5 | 1.00 ± 0.06 | 1.60 ± 0.21 | 1.96 ± 0.37 |
| Y542E | 12 | 1.00 ± 0.17 | 1.54 ± 0.39 | 1.30 ± 0.34 |
| Y542S | 12 | 1.00 ± 0.14 | 0.98 ± 0.09 | 0.66 ± 0.10 |

| Group III: Loss of forskolin stimulation: |
|----------------|----------------|
| L396R | 6 | 1.00 ± 0.06 | 17.79 ± 1.50 | 0.98 ± 0.09 |
| D431Y | 8 | 1.00 ± 0.03 | 20.5 ± 6.23 | 1.56 ± 0.20 |
| K534E/M548V | 11/12 | 1.00 ± 0.06 | 7.70 ± 1.11 | 1.20 ± 0.10 |
| Y542F | 12 | 1.00 ± 0.11 | 3.90 ± 1.00 | 1.61 ± 0.21 |

*aHT22 cells were transfected with pGL3-ERE2-luciferase and individual wild-type or ERα mutants as described in Methods and treated for 24 h with either vehicle, 10 nM estradiol, or 1 μM forskolin. At least three separate experiments with triplicate samples per group were performed. Normalized luciferase activities were calculated as relative to the vehicle-treated controls of individual ER vectors (as 1 fold) and expressed as mean ± SE. The numbers indicate which helices the mutated residues reside as corresponding to the human ERα ligand-binding domain (LBD) reported by Pavlin et al. [49]. DBD, DNA binding domain; S1/S2, two-stranded antiparallel β-sheet. * and † denote significant differences (P < 0.05) as compared to vehicle- and estradiol-treated groups, respectively, using one-way ANOVA and Tukey's honestly significant difference (HSD) post hoc test.
of the entire LBD (ΔE/F) was not degraded by estradiol (54.0 ± 7.4%), and forskolin had no effect on levels of this truncated ERα protein (F, 60.6 ± 5.2% and B, 57.6 ± 6.5%) (Figure 4(b), middle panel). Deletion of the F domain (ΔF) alone or with helix 12 (ΔH12/F) resulted in a dramatic reduction in ERα stability in the absence of estrogen (V, 35.3 ± 7.6% and 32.3 ± 4.8% of untreated controls, respectively), rendering receptors insensitive to ligand-induced degradation (E, 42.4 ± 8.2% and 30.3 ± 4.3%), but higher levels of these truncated receptors were observed with the treatment of forskolin (F, 57.0 ± 10.5% and 73.3 ± 3.7%) or forskolin plus estradiol (B, 61.6 ± 11.1% and 73.0 ± 4.7%) than vehicle-treated groups (P < 0.05) (Figure 4(b), right panels). These data suggest that within the carboxyl-terminal region of ERα, the F domain is required for basal and liganded ERα turnover, while the E domain excluding helix 12 might be indispensable for forskolin-dependent stabilization of the receptor.

To further explore if forskolin-induced ERα stabilization was coupled to its transcriptional activation, we transfected GH3 cells with HA-tagged ERα ΔΔ/Δ carrying L396R or D431Y, the mutants that were not transcriptionally stimulated by forskolin, or S523A, the mutant that prevented ERα phosphorylation by PKA, and then measured the protein levels of transfected ERα in response to estradiol, forskolin, or both. As compared to vehicle-treated controls, levels of ERα L396R (42.3 ± 3.9%), D431Y (45.3 ± 1.2%), and S523A (32.3 ± 5.2%) decreased in the presence of estradiol while forskolin alone stabilized all three ERα mutants (P < 0.05) (Figure 4(c) and S2). Forskolin inhibited ligand-dependent degradation of all three mutated ERα (L396R, 60.1 ± 3.8%; D431Y, 71.5 ± 9.1%; S523A, 51.4 ± 3.7%). Overall, forskolin protection of ERα from degradation seems to be independent of transcriptional activation of the receptor induced by forskolin.

![Figure 3: Forskolin-stimulated ERα transcriptional activity is mediated by specific residues located within the ligand-binding domain. (a) The upper panel shows rat ERα domain structure and the amino-acid sequence of the E domain (amino acids 316–558) with the indication of the alpha helices (underlined or highlighted) and specific residues (bolded) required for forskolin-stimulated ERα transactivation. The helices correspond to those of the human ERα, and S1/S2 is a two-stranded antiparallel β-sheet as reported previously [75]. (b) HT22 cells were similarly transfected with a wild-type or mutated ERα expression vector with single (L396R, D431Y, or Y542F) or dual (K534E/M548V) amino-acid substitutions, followed by treatment of vehicle (V), estradiol (E, 10 nM), or forskolin (F, 1 μM) for 24 h. The luciferase activity (mean ± SEM) was normalized and expressed as the fold stimulation over vehicle-treated cells transfected with wild-type ERα expression vector.

3.5. Forskolin Protection of ERα Protein Does Not Result from Decreased ERα Ubiquitination. Ligand-bound ERα is ubiquitinated, resulting in the degradation of the receptor through the 26S-proteasome pathway; with inhibition of proteasome activity, ubiquitinated ERα can be detected as a ladder of high-molecular-weight conjugates [6, 9, 10, 25]. Therefore, we hypothesized that forskolin might prevent ERα ubiquitination as a means to protect it from degradation. To examine this, GH3 cells transfected with HA-tagged ubiquitin were pretreated with vehicle or MG132, a proteasome inhibitor, followed by the treatment of vehicle, estradiol, forskolin, or both for 1 or 6 h. Cells were lysed, and the extracts were subjected to immunoprecipitation with ERα antibody and then immunoblotted with HA antibody.

After one hour of treatment, low levels of ubiquitinated ERα, revealed by higher molecular weight bands (>183 kD), were observed in the presence of MG132 (Figure 5(a), Lanes 5–8, upper panel), and little ERα was degraded at the same time (Figure 5(a), lower panel). Six hours after the treatment, ERα in the lysate was markedly degraded in the presence of estradiol, but liganded ERα was protected by forskolin or MG132 (Figure 5(b), lower panel). With the presence of MG132, low, noticeable levels of polyubiquitinated ERα...
were detected in control cells, and this was enhanced by estradiol (>80 kD) (Figure 5(b), Lanes 5 and 6). Contrary to our hypothesis, forskolin greatly increased levels of ubiquitinated ERα in the cells in the absence or presence of estradiol (Lanes 3 and 4), and the forskolin-induced ubiquitination was seen more clearly with the pretreatment of MG132 (Lanes 7 and 8) (Figure 5(b)).

We have previously demonstrated that forskolin cannot protect TNFα-induced 1xβα degradation, suggesting that forskolin protection is specific to ERα [8]. To verify that the action of forskolin on ubiquitination was limited to ERα protein, we then examined the effect of forskolin on 1xβα ubiquitination. In the presence of MG132, a basal level of ubiquitinated 1xβα was detected in vehicle-treated cells (Figure 5(c), Lane 5). As 1xβα was protected from TNFα-induced degradation by MG132, a robust increase in the accumulation of ubiquitinated 1xβα was observed (Figure 5(c), Lane 7). Forskolin did not alter the basal or TNFα-induced 1xβα ubiquitination in GH3 cells (Figure 5(c), Lanes 6 and 8), confirming that forskolin-stimulated ubiquitination was ERα-specific, not a global effect.

3.6. ERα Ubiquitination Is Reduced by the K48R Ubiquitin Mutation after All Treatments. Ubiquitination on ubiquitin K48 is essential for the polyubiquitin chain assembly with at least four ubiquitin monomers to mark the substrate proteins for degradation via the proteasome pathway while ubiquitination through other lysine residues of ubiquitin (K11, K29, and K63) alters other biological activities such as protein sorting, translation, and DNA repair [50]. To examine whether forskolin-induced ERα ubiquitination was mediated exclusively by K48, we transfected GH3 cells with vectors containing His6-tagged, wild-type, or K48R ubiquitin; K48R mutant should prevent conjugation of another ubiquitin at the preferred K48 position but not interfere the polyubiquitin chains via other lysines [44]. In the presence of wild-type ubiquitin, treatment with vehicle or estradiol slightly increased ERα ubiquitination, and treatment with forskolin increased both unliganded and liganded ERα ubiquitination substantially (Figure 6(a), left panel). Over-expression of K48R ubiquitin abolished vehicle- and estradiol-induced ERα ubiquitination, and greatly reduced levels of forskolin-stimulated ubiquitinated ERα (Figure 6(a), right panel). This finding confirms that the
formation of K48-linked ubiquitin chains is required for estradiol- and forskolin-mediated ERα ubiquitination.

Besides ubiquitin ligases that add ubiquitin to substrate proteins, deubiquitinases negatively regulate ubiquitination by "trimming" or removing polyubiquitin chains [51]. UCH-L1 is abundant in the mouse pituitary (exclusively in gonadotrophs and lactotrophs) and brain [52]. Thus, we treated GH3 cells, transfected with HA-tagged ubiquitin expression vector, with the inhibitor of UCH-L1 to determine whether this deubiquitinase might contribute to estradiol- and/or forskolin-induced ERα ubiquitination. We found that this treatment slightly enhanced forskolin-induced ubiquitination of unliganded and liganded ERα (Figure 6(b), Lanes 7 and 8). Our result indicates that deubiquitinating enzymes, or at least UCH-L1, may not play a direct role in regulating ERα ubiquitination induced by estradiol or forskolin.

### 3.7. Forskolin-Stimulated ERα Ubiquitination Requires the Carboxyl-Terminal F Domain

We next used rat ERα deletion constructs to assess which specific region was required for estradiol- or forskolin-stimulated ubiquitination. GH3 cells were transfected with HA-tagged ERα expression vector (4 μg). These cells were pretreated with or without MG132 (40 μM) for 1 h, followed by vehicle (V), estradiol (E, 10 nM), forskolin (F, 1 μM), or both (B) for 6 h. Cell lysate was immunoprecipitated (IP) with ERα antibody and then immunoblotted (IB) with HA to detect ubiquitinated ERα (Ubq-ER) (upper panel). These lysates were also subjected immunoblotting (IB) for detecting ERα (lower panel). (b) HA-tagged ubiquitin expressing GH3 cells were similarly pretreated with either vehicle or MG132 for 1 h followed by vehicle (V), estradiol (E), forskolin (F), or both (B) for 6 h. As described above, cell lysates were similarly immunoprecipitated (IP) for ERα and then immunoblotted (IB) for HA to detect ubiquitinated ERα (Ubq-ER) (upper panel) as well as immunoblotting (IB) for ERα in parallel (lower panel). (c) GH3 cells were similarly transfected with HA-tagged ubiquitin expression vector, pretreated with MG132, and then treated with vehicle (V), forskolin (F), TNFα (T, 100 ng/ml), or both (B) for 15 min. Cell lysate was immunoprecipitated (IP) with IκBα antibody and then immunoblotted (IB) with HA to detect ubiquitinated IκBα (Ubq-IκBα) (upper panel). IκBα was also detected by immunoblotting (IB) in these lysates (lower panel).
ERα is required for its ubiquitination elicited by estradiol, forskolin, or both.

3.8. Forskolin Protects ERβ from Estradiol-Dependent Degradation in the Absence of Transcriptional Activation. Although ERβ was not transactivated by forskolin (Figure 1(b)), with a high homology to ERα in the LBD, we tested if forskolin protected ERβ from basal and ligand-induced degradation. We found that in the pretreatment of cycloheximide, unliganded ERβ protein levels were decreased to 48.7 ± 10.0% of control levels, and in response to estradiol, a further reduction (27.3 ± 6.1%) was observed (Figures 8(a) and 8(b)). Unliganded and liganded ERβ were similarly protected from degradation by forskolin (F, 71.1 ± 9.3% and B, 78.0 ± 6.3%, respectively) (Figures 8(a) and 8(b)). In addition, estradiol-bound ERβ appeared to be slightly upshifted, indicating that posttranslational modifications might occur on ERβ after estradiol binding (Figure 8(a), Lanes 2, 4, 6, and 8). This observation suggests that forskolin protection of ERs from degradation seems to be independent of transcriptional activation of the receptor induced by forskolin.
To examine whether forskolin protection of ERβ from degradation was associated with increased ubiquitination, GH3 cells were transfected with HA-ERβ and His6-ubiquitin expression vectors, then pretreated with either vehicle or MG132, followed by vehicle, estradiol, forskolin, or both. In the absence of MG132, estradiol rapidly resulted in ERβ degradation, which made it difficult to detect ubiquitinated ER, whereas ubiquitination of unliganded and liganded ERβ was observed after forskolin treatment alone and with estradiol, respectively (Figure 8(c), Lanes 6–8). Thus, similar to ERα, the ability of forskolin to protect ERβ from degradation does not occur by decreasing ER ubiquitination or depend on stimulation of ER-mediated transcription.

4. Discussion

Our previous study has shown that in ERα-positive, rat GH3 pituitary cells, estradiol- and forskolin-stimulated ER transcriptional activations differ in the time courses of transcriptional activation, coupling with receptor turnover, and responses to proteasome inhibition [8], suggesting distinct mechanisms by which estradiol and forskolin elicit ERα-mediated transcription. ERα transcriptional activity is mediated by the amino-terminal AF-1 (A/B domain) and the carboxy-terminal AF-2 (E/F domain) regions [20, 53, 54], and our deletion mutation experiments demonstrate that similar to estradiol, forskolin stimulation of rat ERα transcriptional activity occurs mainly through the E region, particularly the helix 12 (Figure 2(b)). With the requirement of AF-2, estrogen- and forskolin-dependent activations of ERα are likely to share some common mechanisms. In support of this, overexpression of the p160 coactivators, including steroid receptor coactivator-1 (SRC-1), transcription intermediary factor-2 (TIF2), and receptor-associated coactivator-3 (RAC3), as well as the general coactivators, p300 and CREB-binding protein (CBP), and coactivator-associated arginine methyltransferase 1 (CARM1) enhances ERα-dependent transcription activated by estradiol and CAMP [55, 56]. These findings suggest that estrogen-bound and forskolin-activated ERα may undergo a similar conformational change that forms a binding surface to recruit and interact with coactivators as well as other factors in the basal transcriptional machinery through the helix 12.

Despite the requirement of AF-2 for the responses to estradiol and forskolin, ERα transcriptional activity stimulated by forskolin and estrogen can be differentiated through the mutations of L396R, D431Y, Y542F, or K534E/M548V on ERα LBD (Figure 3(b) and Table 1). Two other mutations, G400V and S464A, were reported earlier to make the human ERα unresponsive to cAMP/PKA while the ligand-dependent transactivation remained intact [55, 57]. These residues are scattered over several helices, including helices 6 (L396), 8 (D431), 10 (S469 in rat; S464 in human), 11 (K534), and 12 (Y542 and M548) as well as the β-sheet (G405 in rat; G400 in human) of the ERα LBD (Figure 3(a)). While normal interactions between ERα and cofactors are suggested to be responsible for forskolin-induced activation of ERα, comparing to the crystal structure of human ERα LBD complexed with estradiol [58], none of these residues, except M548, constitute the hydrophobic groove on the interacting surface of the ERα LBD for coactivator binding. Therefore, we speculate that along with G405 and S469, the two corresponding residues of human ERα G400 and S464, L396, D431, K534, Y542, and M548 might define a novel regulatory surface specific for the interaction of unliganded rat ERα with coactivatory proteins after forskolin stimulation, resulting in distinct transcriptional programs for the ligand-dependent and independent ER activation.

Regarding the molecular mechanism downstream of forskolin stimulation, we have previously shown that forskolin increases ER transcriptional activity exclusively through the cAMP-PKA pathway in ERα-positive pituitary cells [7, 8]. Direct phosphorylation of ERα was originally proposed to mediate the ligand-independent activation by forskolin because PKA phosphorylates human ERα at S236 and S305 [38, 59, 60]. Phosphorylation of S236, located within the DBD, inhibits dimerization and DNA binding of unliganded ERα, and a glutamic acid (S236E), not alanine (S236A), substitution impairs ERα dimerization, which abolishes the estradiol- and PKA-stimulated transactivation [38]. The inhibitory effect of this mutation on ERα transcriptional activity is confirmed at the rat equivalent (S241E) (Table 1). A previous study showed that ligand-independent ERα activation was stimulated by low and intermediate levels of transfected PKA catalytic subunits, but suppressed by high levels, suggesting that the phosphorylation of ERα S236 and its inhibitory effects may occur only at higher levels of cAMP/activated PKA [55]. S305 of human ERα, located in the hinge region, is also phosphorylated by PKA, but an alanine substitution of S305 (S305A) does not abolish ERα-mediated transcription elicited by cAMP or forskolin/IBMX [55, 61]. Apart from S236 and S305, S518 in the LBD is a potential PKA phosphorylation site because of being embedded in a PKA recognition motif. Mutation of the equivalent residue in rat ERα (S523A) had no effect on the estradiol- or forskolin-elicited ERα transcriptional activity (Table 1). On the other hand, L396, D431, K534, Y542, and M548, responsible for the forskolin-stimulated ERα activation, are not phosphorylated residues for PKA or near a consensus substrate motif of PKA. Instead, Carascossa et al. reported that PKA phosphorylated CARM1, which allows the direct binding of CARM1 to unliganded ERα LBD to mediate cAMP activation of ERα [55]. While direct phosphorylation of rat ERα cannot be completely excluded, PKA-dependent phosphorylation of ER-interacting coregulatory proteins, enhancing their recruitment to the receptor, appears to be the molecular basis for the ligand-independent ERα activation by forskolin.

In the present study, we have also observed that the forskolin-stimulated transactivation is specific to rat ERα, but not ERβ (Figure 1(a)), consistent with two previous
studies on human ERα and ERβ [55, 56]. Since AF-2 has the importance for ERα activation in response to forskolin, distinct amino-acid compositions at this region might render the ERs with subtype-specific properties in conveying forskolin signaling. However, the five residues (L396, D431, K534, Y542, and M548) that are required for forskolin stimulation of rat ERα transactivation are all conserved in ERβ (L362, D397, K499, Y507, and M513), suggesting that these amino acids are unlikely to be the molecular determinants of ER subtype-specific activation by forskolin. On the other hand, human ERβ contains an alanine (A) in the position corresponding to S464 in ERα, and alanine substitution (S464A) abolishes the transcriptional response of ERα to cAMP and the interaction of ERα with CARM1 [55]. The serine and alanine residues in the two ER subtypes are conserved from human to rat (S469 in ERα and A435 in ERβ, respectively), so they might be responsible for ERα-specific activation by forskolin. Besides the LBD, the F domains of the rat ERα and ERβ are different in both length (42 versus 28 amino acids) and sequence identity (17%). Replacing the F domain of human ERα with that from ERβ eliminates estradiol-induced transcriptional activity of the receptor at an AP-1 site [62]. Since deletion of the F domain does not abolish forskolin stimulation of rat ERα (Figure 2(b)), the F domain of ERβ might inhibit the receptor from being transcriptionally activated by forskolin, which needs to be elucidated by the removal and replacement of the ERβ F domain.

Besides ERs and their coactivators, the promoter complexity of the reporter gene may also play an important role in defining differential ligand-independent activation between ERα and ERβ [27, 56, 63]. A prior report showed that forskolin/IBMX stimulated the transcriptional activities of both human ERα and ERβ on a complex promoter that contained a 12-O-tetradecanoylphorbol-13-acetate response element (TRE) located upstream to an ERE, and mutation of the TRE abolished the ligand-independent activation of ERβ, but not ERα [56]. This finding may help explain why

![Figure 8: Forskolin protects transfected ERβ from ligand-induced degradation and increases ERβ ubiquitination.](image-url)
forskolin stimulates transcriptional activity of rat ERα, but not ERβ (Figure 1(a)), as we use a simple model reporter with the promoter containing only two EREs upstream of the rat prolactin TATA box to evaluate ER-mediated transcription. In addition, we have also observed that the same forskolin treatment fails to stimulate transfected rat ERα in ER-negative COS cells derived from monkey kidney tissue (not shown). Together, these results suggest that differential forskolin stimulation of ER transcriptional activity between ERα and ERβ might be determined by ER subtypes, promoter contents, and cell contexts.

Estradiol-stimulated ERα transactivation is coupled to increased turnover of the receptor through the ubiquitin-proteasome system [6, 9, 20]. The current study confirms that ligand binding increases ubiquitination and degradation of rat ERα and further demonstrates that both molecular events require the F region (Figures 4(b) and 7(b)). On examining the sequence of the F domain of rat ERα, we discover a PEST-like sequence (amino acid 560–582: RMGVYPEEPSQSLTTTSTSASH) with a high score of 9.23 as predicted by ePestfind (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind). Many short-lived proteins degraded by the ubiquitin-proteasome pathway contain a PEST motif, enriched in proline, glutamate, serine, and threonine, and the PEST sequence serves as a phosphodegron to recruit ubiquitin E3 ligases for protein ubiquitination and degradation. The PEST domain has been shown to mediate IxBα ubiquitination and degradation induced by TNFα [64, 65]. Deletions of the F domain had no effect on ligand-induced human ERα degradation [6, 66]. As compared to the rat, the F domain of human ERα displays only 60% homology and lacks the putative PEST motif, which might be responsible for the discrepancy in the role of the F domain in the regulation of liganded ERα stability between rat and human.

Unlike estradiol, forskolin not only stimulates ERα transcriptional activation without being accompanied by protein degradation but also enhances estradiol-dependent ERα activation with the protection of liganded receptor from proteolysis [8]. Several findings from the current study further support our previous observation, demonstrating that the effect of forskolin on ERα protein stability can be uncoupled from that on transactivation. First, forskolin protects both ERα and ERβ from estradiol-induced degradation, even though only the former is transcriptionally activated by forskolin (Figures 1(b), 4, and 8). Second, two LBD mutants, L396R and D431Y, lacking forskolin-stimulated transcriptional activity, are protected by forskolin from both basal turnover and estradiol-induced degradation (Figures 3(b) and 4(c)). Third, deletion of helix 12 and the F domain disrupts estradiol- and forskolin-induced ERα transactivation while it has no effect on forskolin protection of the receptor from proteolysis (Figures 2(b) and 4(b)). Thus, these observations suggest that forskolin stimulation of ER transcriptional activity and its protection of ER from degradation are possibly mediated by distinct mechanisms.

The ligand-bound ERα and ERβ both are degraded through the ubiquitin-proteasome pathway, so forskolin protection of ERs from degradation prompts us to examine the degree of receptor ubiquitination after estradiol and forskolin treatment. In contrast to our original hypothesis, forskolin drastically increases ubiquitination of ERα and ERβ regardless of the presence or absence of estrogen, which appears to be more robust than that induced by estradiol alone (Figures 5(b) and 8(c)). Forskolin action on ER ubiquitination is protein-specific because the same treatment does not alter TNFα-induced ubiquitination of IxBα (Figure 5(c)). In addition, the F domain is found to mediate both estradiol- and forskolin-stimulated ERα ubiquitination while deletion of the A/B domain does not alter the ubiquitination status of liganded or unliganded ERα (Figure 7). Ubiquitination takes place on the lysine residues of the target proteins [18]. Rat ERα contains 28 lysine residues, but none of those are located within the F domain. Thus, instead of being the substrate site for ubiquitin conjugation, the F region may provide an interaction site that recruits ubiquitin ligases and other components of the ubiquitination machinery. Future investigation will be needed to determine which of the ERα lysines are ubiquitinated in response to estradiol and/or forskolin and to identify the specific ubiquitin ligases involved in these processes.

The signal for the substrate proteins targeted for the proteasomal degradation has been characterized as a polyubiquitination chain that consists at least four ubiquitin monomers conjugated through K48 of ubiquitin, whereas monoubiquitination as well as polyubiquitination through other lysine residues (K11, K29, and K63) within the ubiquitin molecule may involve other cellular functions, such as protein sorting and DNA repair [13, 15–18]. In the current study, the size of ubiquitinated ERα (>114 kD) and requirement of K48 suggest that estradiol and forskolin both stimulate ERα polyubiquitination through K48-linked ubiquitin chains (Figure 6(a)). This agrees with the observation by Iizuka et al., showing that the overexpression of K48R mutant markedly decreased human ERα polyubiquitination [67]. Similar to ERα, the polyubiquitin chains of human ERβ are also linked via K48 [68]. Besides the addition of ubiquitin, deubiquitinating enzymes, including UCH and ubiquitin-specific processing protease (USP), also play important roles in regulation of ubiquitination by removing the ubiquitin from the substrate proteins or the ubiquitin chains [51, 69]. Since inhibition of UCH-L1 slightly enhances ubiquitination of unliganded and liganded ERα, but cannot mimic the action of forskolin (Figure 6(b)), we conclude that UCH-regulated deubiquitination alone may play a small role in forskolin stimulation of ERα ubiquitination. Moreover, the degrees and patterns of ER ubiquitination are different after estradiol and forskolin treatments although both treatments increase polyubiquitination. Increased polyubiquitination at the same sites and/or mono- or polyubiquitination on additional sites within the ER protein may account for such differences.

It has been reported that along with ubiquitin ligases, 19S regulatory components of the proteasome are recruited with ERα on the pS2 promoter, which might lead to the coupling of estrogen-regulated ERα proteolysis and transcription [19, 20]. Our current work showed that forskolin stimulation of ERα transcriptional activity did not require proteasome
activity (Figure 1(c)). Thus, we speculate that uncoupling of forskolin-stimulated ER ubiquitination from degradation might be caused by failure of recruitment of proteasome components. Besides the 26S proteasome, a previous study reported that the lysosome-dependent degradation pathway also contributed to the estradiol-dependent ERα breakdown in MCF-7 cells [70]. Totta and colleagues observed that cytoplasmic ERα was routed to lysosomes and then endosomes in an estradiol-dependent manner, and inhibition of lysosomal function increased liganded ERα accumulation. Interestingly, the lysosome-mediated degradation is not required for ERα-regulated, ERE-containing gene transcription. Thus, forskolin protection of liganded and unliganded ERs from degradation might in part take place in the lysosomes or by preventing ER from being routed to lysosomes.

Similar to transcriptional activity, forskolin protection of ERα from degradation is also mediated through the PKA pathway [8]. Rolli-Derkinderen et al. demonstrated that phosphorylation of RhoA at S188 protected RhoA from ubiquitin-mediated proteasomal degradation [71]. Mutation of the potential PKA phosphorylation site (S523A) in the rat ERα LBD has no effect on receptor turnover (Figure 4(c)), but other PKA phosphorylation sites located in the LBD might mediate the protective effect of forskolin on ERα degradation. Meanwhile, instead of ERα itself, ER-interacting proteins might also be the targets for PKA to increase the stability of the receptor. As mentioned above, several steroid receptor coactivators can be phosphorylated in response to cAMP [55, 72, 73]. Two previous studies have shown that PKA phosphorylates the CARM1 and lysine-specific histone demethylase 1 (LSD1), which induces their recruitment to the unliganded ERα [55, 73]. In addition, PKA-stimulated ubiquitination has been observed in GRIP-1, a steroid receptor coactivator although this was associated with increased protein degradation [74]. Thus, forskolin action on ERα transcriptional activity, protection, and ubiquitination might be contributed by PKA-mediated phosphorylation of ER-interacting proteins rather than ERα itself.

Our studies have characterized several unique features and novel mechanisms for ligand-independent activation and/or protection of ERs by forskolin. Overall, because forskolin-stimulated pathways stabilize both ER subtypes, and because the amount of ER directly correlates to the transcriptional and biological response [46, 75], these signaling pathways will directly impact the ability of specific cells and tissues to respond to ligands as well as to ligand-independent pathways physiologically and pathologically.

Data Availability
The data that support the findings of this study are available on request from the corresponding author, Houng-Wei Tsai (houng-wei.tsai@csulb.edu).

Conflicts of Interest
The authors declare that they have no conflicts of interest.

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Supplementary Materials
Figure S1. Representative immunoblots showing the expression of transfected wild-type (WT) and mutated ERα in ER-negative mouse hippocampal HT22 cells. Cells were transfected with an expression vector of WT or mutated ERα (400 ng) for 24 h. Cells were then lysed, and approximately 30 μg of protein lysates was separated on polyacrylamide-SDS gels, transferred to nitrocellulose membranes, and subjected to immunoblotting with ERα antibody. C, controlled cells transfected with an empty expression vector. Figure S2. Representative immunoblots showing the effects of estradiol, forskolin, or both on protein levels of mutated ERα, including ΔE/F, ΔH12/F, ΔF, and ΔA/B (L396R, D431Y, or S523A), in GH3 cells. Cells, transfected with HA-tagged mutated ERα (400 ng), were pretreated with either vehicle (C) or cycloheximide (+CH, 20 μg/ml) for 30 min. The cycloheximide-pretreated cells were then treated with vehicle (V), estradiol (E, 10 nM), forskolin (F, 1 μM), or both (B) for 6 h. Transfected ERα and endogenous β-actin were detected by immunoblotting with the antibodies against HA and β-actin, respectively. (Supplementary Materials)

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