Phosphorylation-Dependent Sumoylation of Estrogen-Related Receptor α1†

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ABSTRACT: We previously showed that estrogen-related receptor α1 (ERRα1) can compete with estrogen receptor α (ERα) for binding to estrogen response elements (EREs), repressing transcription in the mammary carcinoma cell line MCF-7. Given that ERRα1 can function in the absence of ligands and exists as a phosphoprotein in vivo, we wished to determine sites of phosphorylation involved in regulating its transcriptional activity. Using a combination of electrophoretic mobility shift analysis, phospho-specific fluorescent dye staining, and site-directed mutagenesis, we identified two novel in vivo sites of phosphorylation in the A/B ligand-independent activation domain of ERRα1 at Ser^{19} and Ser^{22}. Inhibition of phosphorylation at amino acid residue 22 did not have a significant effect on ERRα1's transcriptional activity. However, mutation of amino acid residue 19 from serine to alanine enhanced two-fold ERRα1's response to the coactivator GRIP-1. We also identified two sites of sumoylation at Lys^{14} and Lys^{403}. We found that inhibition of sumoylation at Lys^{14} could enhance five-fold ERRα1's response to coactivator GRIP-1. Furthermore, phosphorylation of Ser^{19} enhanced the sumoylation at Lys^{14}. Taken together, we conclude that phosphorylation at Ser^{19} and sumoylation at Lys^{14} within the A/B domain play roles in regulating ERRα1's transcriptional activities via affecting its response to coactivators.

The estrogen-related receptors (ERRs) α, *β*, and *γ* are members of the steroid/thyroid hormone nuclear receptor superfamily that, like other nuclear receptors (NRs), regulate expression of genes via binding specific DNA sequence elements located within promoters (*1*−*3*). The ERRs are structurally similar to most other NRs. They contain a poorly conserved amino-terminal A/B domain and a highly conserved C domain, also known as the DNA-binding domain (DBD),[1] which allows for sequence-specific binding activity. The hinge region D domain enables the C domain to interact with the E/F domain, also known as the ligand-binding domain (LBD) or activation function-2 (AF-2) domain (reviewed in *4*−*6*).

The ERRs share sequence similarity with the estrogen receptors (ERs) α and *β* in their DBDs (reviewed in *4*−*6*). Like the ERs, the ERRs can interact with the p160 family of coactivators, regulating transcription by binding to estrogen response elements (EREs) (*7*, *8*). However, unlike the ERs, the ERRs do not respond or bind to any naturally occurring known ligands, including estrogen. Also unlike the ERs, the ERRs can also bind with high affinity to the extended half-site sequence 5′-TNAAGGTCA-3′, known as an estrogen-related response element (ERRE) (*9*, *10*). Sequence-specific DNA binding of ERRα1 occurs regardless of the presence of exogenous ligand (reviewed in *4*−*6*), activating or repressing transcription in a cell type and promoter-dependent manner (*11*−*15*).

Given that ERRα1 can activate transcription in the absence of ligands, it has been hypothesized that ERRα1's activities are regulated, in part, through post-translational modifications. Barry et al. (*16*) reported that ERRα1's homodimerization and DNA-binding activities can be affected via pathways activated by epidermal growth factor (EGF) and phorbol 12-myristate 13-acetate (PMA). Ariazi et al. (*15*) found that inhibition of the EGFR/ErbB2 (HER-2) signaling pathways can affect both ERRα1's transcriptional activities and extent of phosphorylation. Thus, ERRα1's activities are probably regulated, in part, via changes in phosphorylation at specific amino acid residues within this receptor protein. However, remaining unknown were the specific amino acid residues that become post-translationally modified *in vivo* and the effects of these specific modifications on ERRα1's activities.

Since our laboratory's first report that ERRα1 can downmodulate estrogen signaling in MCF-7 cells (*12*), considerable evidence has appeared that ERRα1's repressor activity plays crucial roles in regulating a variety of physiological processes (e.g., *14*, *17*−*19*). We have pursued the mechanisms regulating ERRα1's repressor activity in the human mammary carcinoma cell line MCF-7. We hypothesized that ERRα1's repressor activity is controlled in these cells, in part, through phosphorylation. Here, we identified amino acid residues 19 and 22 of ERRα1 as major sites of phosphor-

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[1] Abbreviations: AF-2, activation function-2; DBD, DNA-binding domain; EGF, epidermal growth factor; ER, estrogen receptor; ERE, estrogen response elements; ERR, estrogen-related receptor; ERRE, estrogen-related response element; GRIP-1, glucocorticoid receptor-interacting protein-1; LBD, ligand-binding domain; NDSM, negatively charged amino acid-dependent sumoylation motif; PDSM, phosphorylation-dependent sumoylation motif; Ppase, phosphatase; SENP1, sentrin-specific protease 1; SUMO, small ubiquitin-like modifier.

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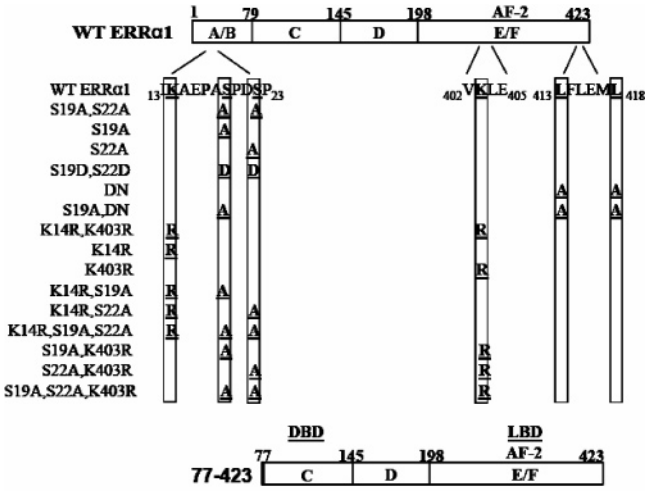

FIGURE 1: Schematic representations of WT ERRα1 and the substitution and deletion variants of it studied here. The bold letters A−F in WT ERRα1 denote the domains commonly found in nuclear hormone receptors. Numbers indicate amino acid residue numbers of the protein. The sequences of residues 11−23, 402−405, and 413−418 for the WT and mutant variants are shown, with the substituted amino acid residues indicated by underlined, bold letters.

ylation in these cells, with inhibition of phosphorylation at Ser^{19} increasing ERRα1's transcriptional response to the coactivator glucocorticoid receptor-interacting protein-1 (GRIP-1) via its AF-2 domain.

Recently, Hietakangas et al. identified a phosphorylation-dependent sumoylation motif (PDSM) that consists of I/L/V-K-X-E-X-X-S-P, with phosphorylation of a proline-directed serine promoting the sumoylation of an upstream lysine (*20*). ERRα1's Ser^{19} is part of such a motif, with a potential sumoylation site at Lys^{14}, which has previously been noted to be potentially part of a synergy control motif (*21*). We show here that ERRα1 was sumoylated at Lys^{14} and Lys^{403}. Inhibition of sumoylation at Lys^{14}, but not at Lys^{403}, led to a greater response to GRIP-1 than either WT ERRα1 or $ERR\alpha1_{S19A}$. Furthermore, the efficiency of sumoylation at Lys^{14} was increased by phosphorylation at Ser^{19}. Thus, we conclude that ERRα1's transcriptional activities can be regulated, in part, via phosphorylation-dependent sumoylation within the amino-terminal A/B domain affecting ERRα1's response to coactivators.

MATERIALS AND METHODS

Plasmids. The ERRα1 mutants generated in this study are shown schematically in Figure 1. Plasmid pcDNA3-hERRα1 encodes wild-type human ERRα1 cloned into the pcDNA 3.1 myc-His (A−) vector (Invitrogen) at the *Bam*HI and *Eco*RI restriction sites. To ensure efficient initiation and termination of translation of the ERRα1 open reading frame, the cloning was performed using the primers 5′-ccggaattcGCCACCATG**AG**CAGCCAGGTGGTGGGC-3′ (lower-case letters indicate an *Eco*RI site, underlined letters indicate the translation initiation codon, and bold letters indicate bases altered to optimize translation initiation while maintaining coding of WT ERRα1 protein) and 5′-cgcggatccTCAGTCCATCATGGCCTCGAGCAT-3′ (lower-case letters indicate a *Bam*HI site, and underlined letters indicate the translation termination codon). Amino acid substitution mutants of ERRα1 were created through site-directed mutagenesis of pcDNA3-hERRα1 by standard PCR cloning methods. The plasmid pcDNA3-GRIP-1 encodes the coactivator GRIP-1 (*22*). Plasmid pTATA-ffLuc, containing a basal promoter driving expression of firefly luciferase, and plasmid pERE-(5×)-ffLuc, containing five tandem copies of the consensus palindromic ERE, were constructed as previously described (*15*).

Plasmids used for sumoylation assays consisted of pcDNA-6xmycUbc9 (generous gift from Dr. Shigeki Miyamoto), pcDNA3-HA-SUMO-3 (*23*, a kind gift from Dr. Jorge Iñiguez-Lluhí), and pcDNA3-FH-SENP1 (*24*, a kind gift from Dr. Peter O'Hare).

Cells. MCF-7/WS8 (referred to here as MCF-7) is a clonal derivative of the human mammary carcinoma cell line MCF-7 that maintains estrogen responsiveness (*25*, *26*); it was a gift from Dr. Craig Jordan. These cells were cultured in DMEM/F12 medium containing 10% whole fetal bovine serum (FBS), 6 ng/mL insulin, 2 mM L-glutamine, 100 μM nonessential amino acids, and 100 U of penicillin and streptomycin per mL at 37 °C and 5% CO_2.

Transient Transfections and Luciferase Assays. Twenty-four hours prior to transfection, MCF-7 cells were seeded in 24-well plates at approximately 50 000 cells/well. The next day, the cells were fed fresh medium in the presence or absence of 10^{-7} M fulvestrant (ICI 182,780; AstraZeneca). The cells were cotransfected using TransIT LT1 reagent (Mirus) with (i) 0.20 μg of pERE(5×)-ffLuc or pTATA-ffLuc as an external control; (ii) 0.15 μg of pcDNA3 ERRα1, pcDNA3 $ERR\alpha1_{77-423}$, pcDNA3 $ERR\alpha1_{S19A,S22A}$, pcDNA 3 $ERR\alpha1_{S19A}$, pcDNA3 $ERR\alpha1_{S22A}$, pcDNA3 $ERR\alpha1_{S19D,S22D}$, pcDNA3$ERR\alpha1_{DN}$, pcDNA3$ERR\alpha1_{S19A,DN}$, pcDNA3$ERR\alpha1_{K14R}$, pcDNA3, pcDNA3$ERR\alpha1_{K403R}$, pcDNA3 $ERR\alpha1_{K14R,K403R}$, pcDNA3$ERR\alpha1_{K14R,S19A}$, pcDNA3$ERR\alpha1_{K14R,S22A}$, pcDNA3-$ERR\alpha1_{K14R,S19A,S22A}$, or their empty parental vector, pcDNA3; and (iii) 0.20 μg of pGRIP-1 or its empty parental vector, pcDNA3. Cells were harvested 48 h post-transfection and assayed for luciferase activity. Data were internally normalized to protein concentration and externally normalized to luciferase activity in the cells cotransfected in parallel with the basal TATA reporter. Transfections were performed in triplicate, with each experiment repeated at least three times on different days. Data shown were obtained from representative experiments. To ensure the observed activation by GRIP-1 was not, in reality, due to squelching of transcription by pcDNA3, these assays were also performed with equimolar amounts of GRIP-1 and empty parental vector making up the mass difference by addition of promoterless pGL3-Basic DNA (Invitrogen); the data obtained were similar to that observed using equal microgram amounts of pGRIP-1 vs pcDNA3 (data not shown).

Immunoblotting and Phosphatase Treatment. Twenty-four hours after seeding, MCF-7 cells were transfected with 3.0 μg of the indicated DNA per 10 cm dish. Forty-eight hours later, whole-cell extracts were prepared by three cycles of freezing and thawing in lysis buffer [50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, 1:100 protease inhibitor cocktail (Calbiochem), and 1:100 phosphatase inhibitor cocktail (Calbiochem)]. Lysates were cleared by centrifugation at 16 000*g* for 15 min at 4 °C. Where indicated, 5 μg of protein extract was incubated with λ phosphatase (New England Biolabs) in a 10 μL reaction

volume at 30 °C for 15 min. The proteins were separated by electrophoresis at 100 V by 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) for 3 h to resolve differentially phosphorylated forms of ERRα1. Proteins were transferred to a nitrocellulose membrane and probed with the ERRα1-specific polyclonal antibody YC2 (*10*). An anti-rabbit IgG horseradish peroxidase-linked secondary antibody (Amersham) and enhanced chemiluminescence substrate (Roche) were used for detection of the signal.

Immunoprecipitation and Phosphostaining. Immunoprecipitation of ERRα1 from whole-cell extracts was performed using Mag-prep anti-mouse IgG beads (Novagen). An ERRα1-specific mouse monoclonal antibody, 1ERR87 (a generous gift from Dr. Richard Burgess), was bound to the beads by incubation for 1 h at 4 °C. Immunoprecipitation was performed by incubation of whole-cell extracts with the antibody-containing beads for 3 h at 4 °C. Proteins in the immunoprecipitate were separated by 12% SDS-PAGE. Phosphoproteins in the gel were fixed by incubation overnight in 50% methanol and 10% acetic acid and visualized by incubation with Pro-Q Diamond phospho-specific fluorescent dye (Invitrogen/Molecular Probes) according to the manufacturer's instructions. Immunoblots were performed on aliquots of the same immunoprecipitates to normalize for relative amounts of ERRα1 protein. The phospho-stained gel shown in Figure 4A is a representative one from experiments performed on three occasions. Relative intensities of the phosphostained bands from the three separate experiments were quantified using ImageQuant (Molecular Dynamics).

Electrophoretic Mobility Shift Assays (EMSAs). EMSAs were performed using whole-cell extracts from MCF-7 cells overexpressing the indicated ERRα1 proteins. The double-stranded oligonucleotide 5′-TAAGCTTAGGTCACAGTGACCTAAGCTTA-3′ was used as the ^{32}P-radiolabeled ERE probe. Approximately 5 μg of whole-cell extract was preincubated on ice for 20 min in a 16 μL reaction containing 20 mM HEPES, pH 7.4, 1 mM dithiothreitol, 100 mM NaCl, 10% glycerol (v/v), and 4 μg of poly(dI-dC). The radiolabeled ERE probe was added to the reaction mixture, and incubation was continued at room temperature for an additional 15 min. Samples were loaded onto a nondenaturing 5% polyacrylamide gel and electrophoresed at 200 V for 2 h at 4 °C.

In Vivo Sumoylation. Twenty-four hours after seeding in 10 cm dishes, MCF-7 cells were transfected with 1 μg of the indicated ERRα1 expression plasmid, 1 μg of pcDNA-6XmycUbc9, and 1 μg of pcDNA3-HA-SUMO-3. Whole-cell extracts were prepared in lysis buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 10% glycerol, 2 mM EDTA, 50 mM NaF, 1:100 protease inhibitor cocktail (Calbiochem), 1:100 phosphatase inhibitor cocktail (Calbiochem), and 20 mM *N*-ethylmaleimide (NEM)]. Prior to immunoprecipitation, lysates were treated with 40 mM DTT. Immunoprecipitations and immunoblots were performed as described above. All in vivo sumoylation experiments were performed on two or more separate occasions.

In Vitro Sumoylation. ERRα1 was synthesized in vitro using the TNT T7 coupled reticulocyte lysate system (Promega) in the presence of ^{35}S-methionine. In vitro sumoylation assays were performed with 2 μL of in vitro synthesized ERRα1 and SUMO-1, -2, and -3 conjugation kits (Boston Biochem) according to the manufacturer's protocol. Reactions were incubated at 37 °C for 60 min and terminated by addition of SDS loading buffer. Products were separated by 12% SDS-PAGE, fixed in acetic acid, treated with the liquid scintillant PPO, dried, and subjected to autoradiography.

RESULTS

N-Terminal Domain of ERRα1 Contains Repressor Activity. Our laboratory had previously shown that while ERRα1 poorly activates transcription in MCF-7 cells it can function as a fairly strong activator on the same promoter in a variety of other cell lines (e.g., BT-474) (*12*, *15*). The amino-terminal domain of some nuclear receptors is known to contribute to regulation of repressor activity (*21*, *27*−*29*). To examine whether this might be the case with ERRα1 as well, we generated ERRα1$_{77-423}$, a deleted variant of ERRα1 lacking the amino-terminal A/B domain. We cotransfected MCF-7 cells in parallel with (i) a luciferase reporter plasmid containing five tandem EREs or only a basal TATA promoter as an external control and (ii) an expression plasmid containing WT ERRα1, ERRα1$_{77-423}$, or their parental empty vector, pcDNA3, as a control for endogenous background effects. As expected (*12*), overexpression of WT ERRα1 in MCF-7 cells led to an approximately eight-fold repression of endogenous ERα-activated transcription; on the other hand, overexpression of ERRα1$_{77-423}$ led to only a two-fold repression of ERα-activated transcription (Figure 2A).

To examine the activation of transcription by ERRα1 in the same cell context in the absence of endogenous ERα, we repeated the cotransfection experiment in MCF-7 cells, performing it in the presence of (i) the complete antiestrogen, ICI 182,780 (also known as fulvestrant), to inhibit endogenous ERα activity and (ii) plasmid expressing the coactivator GRIP-1. Because ERRα1 functions as a weak activator in this cell line (*12*), overexpression of GRIP-1 had, as expected, only a minimal effect on the activity of either endogenous or exogenous WT ERRα1. However, overexpression of the ERRα1$_{77-423}$ mutant led to a two-fold and 24-fold activation above background levels of ERE-regulated transcription in the absence and presence of the GRIP-1 expression plasmid, respectively (Figure 2B). Immunoblot analysis indicated that the accumulation of ERRα1$_{77-423}$ in the cells was less than that of WT ERRα1 (Figure 2C), with this reduced amount of receptor still able to bind a consensus ERE as measured by an EMSA (Figure 2D). Semiquantitative immunoblot analysis indicated that ERRα1$_{77-423}$ was expressed at approximately one-third the amount of WT ERRα1 (data not shown). Transfection of cells with a lesser amount of WT ERRα1 expression plasmid so that WT ERRα1 accumulated in the cells to a similar level to that of ERRα1$_{77-423}$ did not enhance its response to GRIP-1 (data not shown). Thus, despite lower accumulation than WT ERRα1, ERRα1$_{77-423}$ exhibited a greatly enhanced response to coactivator GRIP-1 (Figure 2B). Therefore, we conclude that the amino-terminal domain of ERRα1 contains repressor activity that affects ERRα1's ability to respond to GRIP-1.

ERRα1 Is Phosphorylated at Ser^{19} and Ser^{22}. ERRα1 is known to be a phosphoprotein in vivo (*9*, *15*, *16*). To assay for phosphorylation, we treated whole-cell extracts containing overexpressed WT ERRα1 with λ phosphatase (ppase), a

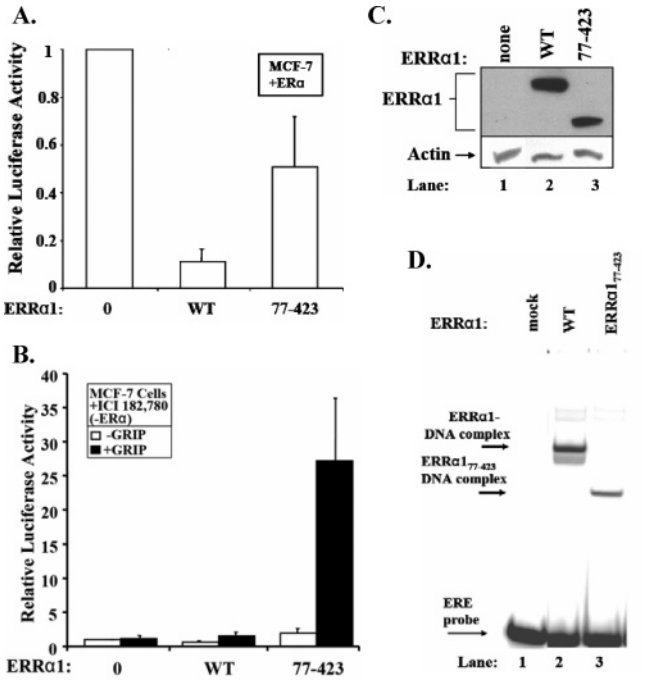

FIGURE 2: Amino-terminal domain of ERRα1 contains repressor activity. (A) Repression and (B) activation activity of WT ERRα1 and amino-terminal deleted variant of ERRα1$_{77-423}$. MCF-7 cells were cotransfected in parallel with (i) a 5× ERE-regulated or basal TATA luciferase reporter, (ii) the indicated ERRα1 expression vector, and (iii) a GRIP-1 expression plasmid or its parental empty vector. Cells were incubated for 48 h in medium containing whole serum in the (A) absence or (B) presence of 10^{-7} M ICI 182,780. Cells were assayed for luciferase activity, with normalization both internally to total protein and externally to activity of the basal TATA reporter transfected in parallel. Data shown are means ± standard deviations of results obtained from a representative experiment performed in triplicate on three different occasions. (C) Immunoblot of whole-cell extracts made from MCF-7 cells transfected with the indicated ERRα1 expression plasmids. Five percent of each protein extract was separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with 1ERRα21 monoclonal antibody or an actin-specific antibody. (D) EMSAs showing DNA-binding activities of WT ERRα1 and ERRα1$_{77-423}$. Whole-cell extracts from MCF-7 overexpressing the indicated ERRα1 expression plasmids were incubated with a radiolabeled ERE probe. The protein−DNA complexes were analyzed by 5% PAGE.

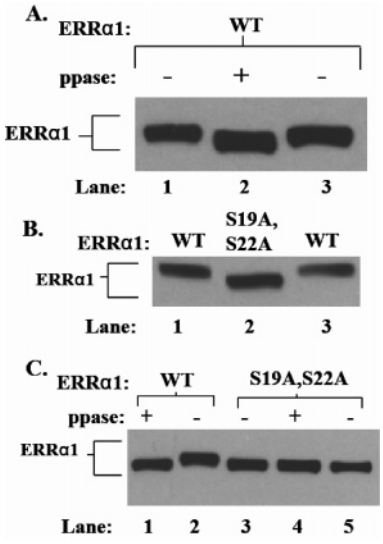

FIGURE 3: WT ERRα1 is a phosphoprotein in MCF-7 cells. (A) Effect of λ phosphatase treatment on WT ERRα1's electrophoretic mobility. Five micrograms of whole-cell extract made from MCF-7 cells transfected with the WT ERRα1 expression plasmid was treated with λ phosphatase (lanes 1 and 3) and analyzed alongside 5 μg of untreated extract (lane 2) by 12% SDS-PAGE. The protein was transferred to a nitrocellulose membrane and immunoblotted with an ERRα1-specific polyclonal antibody. (B) ERRα1$_{S19A,S22A}$ migrates faster than WT ERRα1. Proteins in whole-cell extracts made from MCF-7 cells containing overexpressed WT ERRα1 (lanes 1 and 3) or the ERRα1$_{S19A,S22A}$ mutant variant (lane 2) were separated by electrophoresis and probed as described in panel A above. (C) WT ERRα1 is primarily phosphorylated at amino acid residues 19 or 22 in vivo. Whole-cell extract made from MCF-7 cells containing overexpressed WT ERRα1 or the ERRα1$_{S19A,S22A}$ mutant variant were treated with λ phosphatase where indicated and analyzed alongside corresponding untreated extracts as described above.

phosphatase known to remove phosphate groups from serines, threonines, and tyrosines. Ppase-treated whole-cell extract was electrophoresed alongside untreated whole-cell extracts in an SDS 12% polyacrylamide gel. ERRα1 was detected by immunoblotting with an ERRα1-specific antibody. As expected, the ppase-treated WT ERRα1 migrated faster in the gel than did the untreated WT ERRα1 (Figure 3A, lane 2 vs lanes 1 and 3), confirming that ERRα1 exists as a phosphoprotein in MCF-7 cells.

Given that ERRα1 exists as a phosphoprotein and the amino-terminal domain of ERRα1 contributes to regulation of its transcriptional activity, we looked for sites of phosphorylation in the N-terminal domain that may play roles in this regulation. The consensus sequence for proline-directed kinases, S/T-P, is frequently present and phosphorylated in nuclear receptors (reviewed in *30*). This consensus sequence is present in the amino-terminal domain of ERRα1 only at amino acid residues 19 and 22. To test whether these two amino acid residues are, in fact, phosphorylated in MCF-7 cells, we generated the double substitution mutant ERRα1$_{S19A,S22A}$ containing alanines in place of serines at amino acid residues 19 and 22. ERRα1$_{S19A,S22A}$ migrated faster than WT ERRα1 by SDS-PAGE (Figure 3B, lane 2 vs lanes 1 and 3). Contrary to WT ERRα1, ERRα1$_{S19A,S22A}$'s mobility was unaffected by treatment with λ ppase (Figure 3C, lane 4 vs lanes 3 and 5) and was similar to the mobility of the phosphatase-treated WT ERRα1 (Figure 3C, lanes 1−3).

To determine which of these amino acid residues were phosphorylated, we also generated the single amino acid substitution mutants ERRα1$_{S19A}$ and ERRα1$_{S22A}$. These mutants were overexpressed in MCF-7 cells in parallel with WT ERRα1 and ERRα1$_{S19A,S22A}$. The mutant ERRα1s and WT ERRα1 were immunoprecipitated, separated by SDS-PAGE, and analyzed for phosphorylation by staining with the phospho-specific fluorescent dye, Pro-Q Diamond. Pro-Q Diamond allows for the visualization of phosphorylation under physiological, steady-state conditions. As expected, the ERRα1$_{S19A,S22A}$ protein displayed a marked decrease in staining with the phospho-specific dye, down to near endogenous ERRα1 background levels (Figure 4A, lane 4 vs lanes 1 and 2). On the other hand, both of the single mutants retained phospho-specific staining well above background (Figure 4A, lanes 5 and 6). Semiquantitative analysis of the phosphostaining from three independent experiments indicated that ERRα1$_{S19A}$ and ERRα1$_{S22A}$ exhibited approximately 50 and 85%, respectively, of the phosphostaining

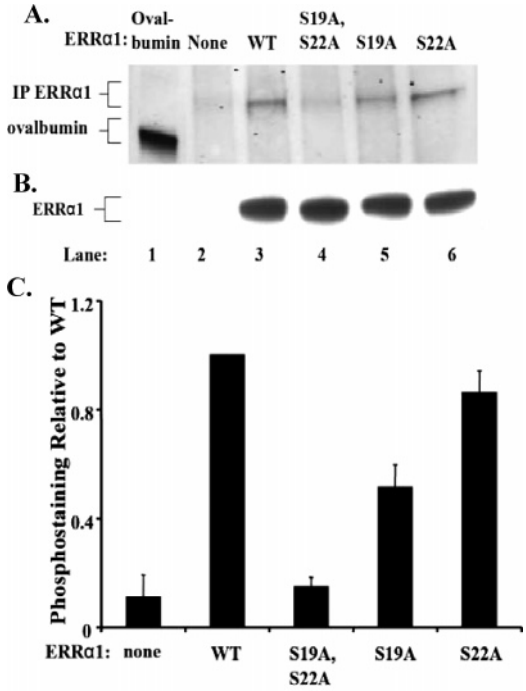

FIGURE 4: ERRα1 is phosphorylated on amino acid residues 19 and 22 in vivo in MCF-7 cells. (A) Phosphostain of WT ERRα1 and phosphomutant variants. The indicated overexpressed ERRα1s from MCF-7 whole-cell extracts were immunoprecipitated with an ERRα1-specific monoclonal antibody, subjected to 12% SDS-PAGE, and stained with the Pro-Q diamond phosphospecific fluorescent dye. Ovalbumin was run in lane 1 as a positive control. Lanes 2−6 contained immunoprecipitates of WCE containing empty vector plasmid or the indicated ERRα1 expression plasmid. (B) Immunoblots performed with an ERRα1-specific polyclonal antibody on a portion of each immunoprecipitate from panel A. (C) Quantification of phosphorylation intensity. Relative intensities of the phosphostained proteins were quantified using ImageQuant. Band intensities were corrected for average background and normalized relative to WT ERRα1 in lane 3. Data shown are the means ± SDs of results obtained from three experiments.

observed with WT ERRα1 (Figure 4C). Immunoblot analysis of the immunoprecipitates with an ERRα1-specific polyclonal antibody showed that all of the proteins had accumulated to similar levels in the cells (Figure 4B). Thus, the differential phosphostaining was due to differences in the extent of phosphorylation of the mutant and WT ERRα1 proteins. We conclude that Ser^{19} and Ser^{22} are the major sites of phosphorylation of ERRα1 in MCF-7 cells when grown under the standard culture conditions as described in the Materials and Methods.

Phosphorylation at Ser19 Regulates ERRα1's Transcriptional Activities. To examine whether phosphorylation at Ser^{19} or Ser^{22} affects ERRα1's transcriptional activity, we used the luciferase reporter assay described above with WT ERRα1 and its phosphomutant variants. We also generated and tested, likewise, $ERR\alpha1_{S19D,S22D}$, a mutant variant in which Ser^{19} and Ser^{22} were replaced with aspartic acids to mimic constitutive phosphorylation at these sites. As expected, overexpression of WT ERRα1 led to an approximately seven-fold repression of ERα-activated transcription (Figure 5A). When GRIP-1 was not overexpressed, all ERRα1 mutant variants repressed ERα-activated transcription to a level similar to the level observed with overexpressed WT ERRα1 (Figure 5A, white bars). However, when GRIP-1 was overexpressed, $ERR\alpha1_{S19A,S22A}$ and

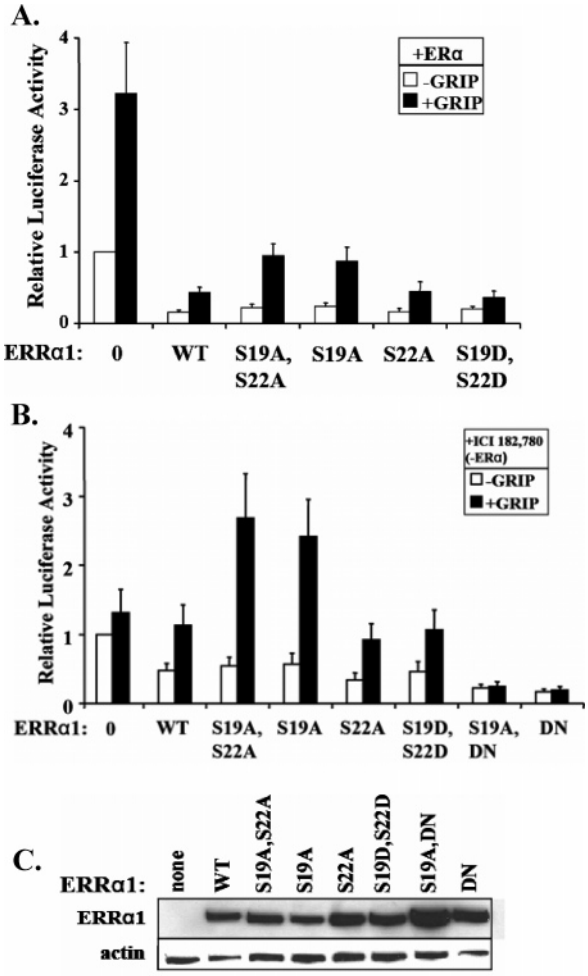

FIGURE 5: Inhibition of phosphorylation at Ser^{19} increases ERRα1's response to coactivator GRIP-1. MCF-7 cells were cotransfected in parallel with (i) an ERE-regulated or basal TATA luciferase reporter as an external control, (ii) the indicated ERRα1 expression plasmid, and (iii) GRIP-1 or its empty parental plasmid. Cells were incubated for 48 h in medium in the absence (A) or presence (B) of 10^{-7} ICI 182,780. After incubation, the cells were harvested and assayed for luciferase activity as described in the legend to Figure 2. (C) Immunoblots of whole-cell extracts overexpressing the indicated ERRα1 expression plasmids studied in panels A and B above were probed with an ERRα1-specific monoclonal antibody.

$ERR\alpha1_{S19A}$ were approximately two-fold less effective at repressing ERα-activated transcription than was WT ERRα1, whereas $ERR\alpha1_{S22A}$ and $ERR\alpha1_{S19D,S22D}$ repressed ERE-dependent transcription at a level similar to WT ERRα1 (Figure 5A, black bars). Thus, we conclude that phosphorylation at amino acid residue 19 of ERRα1 contributes to regulating GRIP-1-dependent activated transcriptional activation by ERRα1.

We also examined the effect of phosphorylation at amino acid residues 19 and 22 on ERRα1's activation activities in the absence of ERα by incubating the cells in the presence of the anti-estrogen ICI 182,780 as described above. WT ERRα1, $ERR\alpha1_{S22A}$, and $ERR\alpha_{S19D,S22D}$ exhibited no ERE-dependent activation above the background observed in the absence of exogenous ERRα1 regardless of whether GRIP-1 was overexpressed (Figure 5B). On the other hand, although the activities of $ERR\alpha1_{S19A,S22A}$ and $ERR\alpha1_{S19A}$ were similar to WT ERRα1 in the absence of overexpressed GRIP-1 (Figure 5B, white bars), $ERR\alpha1_{S19A,S22A}$ and $ERR\alpha1_{S19A}$

activated ERE-dependent transcription approximately two-fold above WT ERRα1 in the presence of GRIP-1 (Figure 5B, black bars).

To begin to examine if these changes in ERRα1's activity were due to direct intramolecular interactions between ERRα1 and GRIP-1, we generated the ERRα1 dominant-negative mutant variant $ERR\alpha1_{DN}$ in which the two leucines at amino acid residues 413 and 418 have been substituted with alanines, thereby inhibiting coactivator binding to ERRα1's AF-2 domain (*12*, *31*). The dominant-negative mutation was also subcloned into the S19A mutant to generate the triple substitution mutant $ERR\alpha1_{S19A,DN}$. If our hypothesis is valid, the increased response to coactivator GRIP-1 observed with $ERR\alpha1_{S19A}$ should be eliminated when the DN mutation is present as well. This is what was observed (Figure 5B). Immunoblot analysis indicated that all of the mutant variants accumulated in the cells to levels similar to WT ERRα1 (Figure 5C); the DN mutation actually led to enhanced accumulation, a phenomenon often seen with coactivator binding mutants (e.g., *32*−*34*). Thus, we conclude that the absence of phosphorylation at amino acid residue 19 enhances the ability of ERRα1 to activate ERE-dependent transcription via response to GRIP-1 at ERRα1's carboxy-terminal end.

Lys[14] and Ser[19] Are Part of a Putative PDSM. Recently, Hietakangas et al. (*20*) identified a PDSM that consists of I/L/V-K-X-E-X-X-S-P, with phosphorylation of a proline-directed serine promoting the sumoylation of an upstream lysine within the sumoylation consensus sequence I/L/V-K-X-E. ERRα1's Ser[19] is part of such a motif, with a potential sumoylation site at Lys[14]. We also noticed another sumoylation consensus motif not within a PDSM at Lys[403]. Sumoylation of a transcription factor often correlates with repression activity (reviewed in *35*). Thus, the inhibitory effect of phosphorylation at Ser[19] could be due to the promotion of sumoylation at Lys[14] of ERRα1. To begin to test this hypothesis, we constructed and assayed as described above plasmids expressing $ERR\alpha1_{K14R,K403R}$, $ERR\alpha1_{K14R}$, and $ERR\alpha1_{K403R}$, mutant variants of ERRα1 in which the potentially sumoylated lysines are replaced with arginines. Strikingly, the $ERR\alpha1_{K14R,K403R}$ and $ERR\alpha1_{K14R}$ mutant variants activated transcription approximately five-fold above WT ERRα1 in the presence of overexpressed GRIP-1 (Figure 6A). On the other hand, the $ERR\alpha1_{K403R}$ mutant exhibited activity similar to WT ERRα1. Immunoblot analysis showed that the mutant proteins accumulated to levels similar to WT ERRα1 (Figure 6B). Thus, we conclude that Lys[14] plays a central role in regulating GRIP-1-mediated transcriptional activation by ERRα1.

One hypothesis is that sumoylation of Lys[14] is regulated, in part, by phosphorylation at Ser[19], with the two-fold increase in activation seen with $ERR\alpha1_{S19A}$ and $ERR\alpha1_{S19A,S22A}$ being due primarily to its ability to enhance efficiency of sumoylation, without it being absolutely required for sumoylation to occur. If this hypothesis is valid, we would predict that mutant variants defective in both sumoylation at Lys[14] and phosphorylation at Ser[19] or Ser[22] would exhibit a phenotype similar to $ERR\alpha1_{K14R}$ rather than showing synergistic effects. The former is what we observed; that is, the mutant variants, $ERR\alpha1_{K14R}$, $ERR\alpha1_{K14R,S19A}$, $ERR\alpha1_{K14R,S22A}$, and $ERR\alpha1_{K14R,S19A,S22A}$ all exhibited an approximately five-fold increase in GRIP-1-mediated activation relative to WT ERRα1 (Figure 6C). Immunoblot analysis showed that the mutant variant proteins accumulated to levels similar to WT ERRα1 (Figure 6D). Thus, we conclude that Lys[14], a putative sumoylation site adjacent to Ser[19], plays a major role in regulating ERRα1's response to coactivator, with phosphorylation at Ser[19] likely regulating ERRα1's activity via its effects on sumoylation.

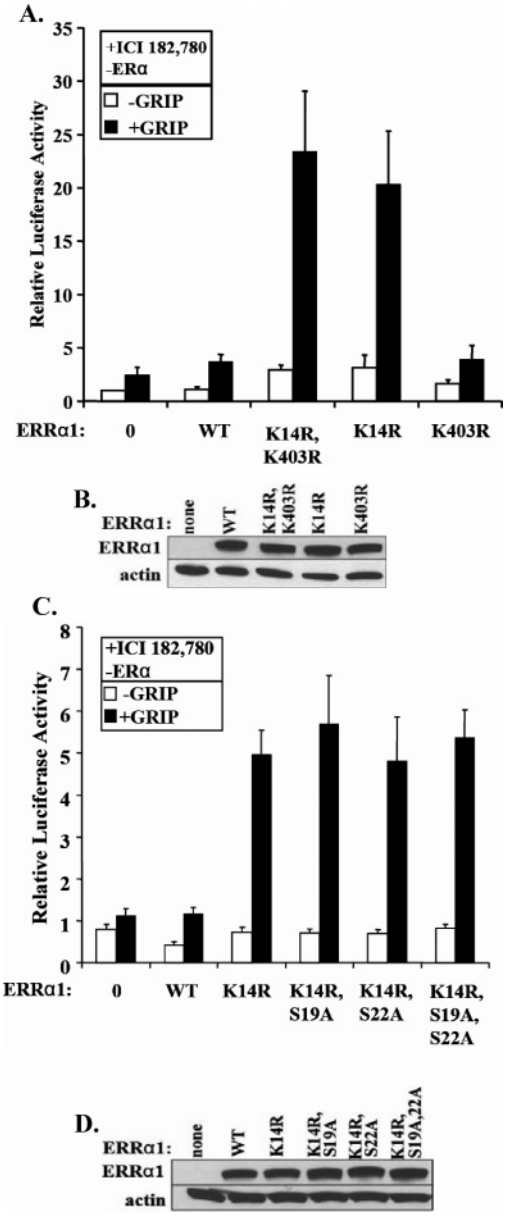

FIGURE 6: Mutation of Lys[14] on ERRα1 increases its response to coactivator GRIP-1. (A and C) MCF-7 cells were cotransfected in parallel with (i) an ERE-regulated or basal TATA-regulated luciferase reporter as an external control, (ii) the indicated ERRα1 expression plasmid, and (iii) GRIP-1 or its empty parental plasmid. Cells were incubated for 48 h in medium containing whole FBS in the presence of 10^{-7} ICI 182,780. After incubation, the cells were harvested and assayed for luciferase activity as described in the legend to Figure 2. (B and D) Immunoblots of whole-cell extracts overexpressing the indicated ERRα1 expression plasmids studied in panel A were probed with an ERRα1-specific monoclonal antibody.

ERRα1 Is Sumoylated at Lys[14] and Lys[403]. To test directly whether ERRα1 can, indeed, be sumoylated in vivo, we cotransfected cells with plasmids expressing (i) Ubc9, the only conjugating enzyme for sumoylation of proteins in cells

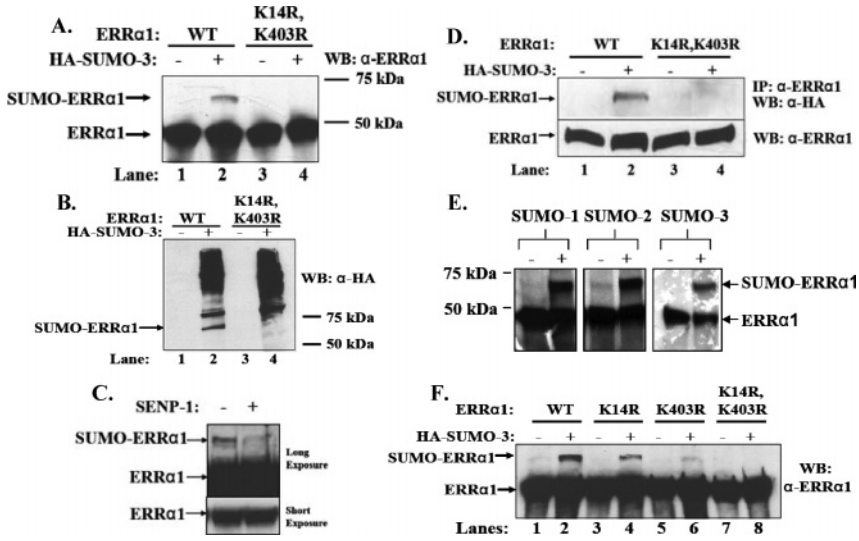

FIGURE 7: ERRα1 is sumoylated at Lys[14] and Lys[403]. (A) Immunoblot analysis of ERRα1 synthesized in vivo in the absence and presence of pcDNA-HA-SUMO-3. MCF-7 cells were cotransfected with (i) the indicated ERRα1 expression plasmid, (ii) pcDNA-6xmycUbc9, and (iii) pcDNA-HA-SUMO-3 or its parental vector. Cells were harvested 48 h later, and whole-cell extracts were generated. Proteins from 5% of each whole-cell extract were separated by 12% SDS-PAGE, transferred to a membrane, and probed with an ERRα1-specific polyclonal antibody. (B) Immunoblot analysis of cellular sumoylated proteins. The nitrocellulose membrane from panel A was stripped and reprobed for sumoylated proteins with an HA-specific polyclonal antibody. (C) Immunoblot analysis of SENP1 effect on ERRα1 sumoylation. MCF-7 cells were cotransfected with (i) WT ERRα1 expression plasmid, (ii) pcDNA-6xmycUbc9, (iii) pcDNA-HA-SUMO-3, and (iv) pcDNA3-FH-SENP1. Proteins from 5% of each whole-cell extract were subjected to immunoblot analysis with an ERRα1-specific polyclonal antibody. (D) Sumoylation of ERRα1. The ERRα1 proteins present in the whole-cell extracts generated in panel A were immunoprecipitated with an ERRα1-specific monoclonal antibody, separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with an HA-specific polyclonal antibody. Afterward, the immunoblot was stripped and reprobed with an ERRα1-specific polyclonal antibody. (E) In vitro sumoylation of ERRα1. In vitro sumoylation assays were performed with in vitro-synthesized [^{35}S]-labeled WT ERRα1 at 37 °C for 60 min. Reaction products were separated by 12% SDS-PAGE, dried, and subjected to autoradiography. (F) Identification of sites of sumoylation. MCF-7 cells were cotransfected with (i) the indicated ERRα1 expression plasmid, (ii) pcDNA-6xmycUbc9, and (iii) pcDNA-HA-SUMO-3 or its parental plasmid. Cells were harvested 48 h after transfection, and whole-cell extracts were generated. Proteins from 5% of each whole-cell extract were subjected to immunoblot analysis with an ERRα1-specific polyclonal antibody.

that is often rate-limiting (reviewed in *36*), (ii) WT or the indicated mutant variant of ERRα1, and (iii) HA-tagged SUMO-3 where indicated. Whole-cell extracts were generated in the presence of NEM to inhibit desumoylases, and the proteins were separated by SDS-PAGE. Sumoylation of a protein typically correlates with an approximately 20 kDa increase in mobility. When probed for ERRα1, a novel band was observed with this expected increase in mobility (Figure 7A, lane 2). The band was no longer observed when the cells were cotransfected, instead, with ERRα1$_{K14R,K403R}$ (Figure 7A, lane 4).

To confirm that this novel band was, indeed, sumoylated ERRα1, we stripped and reprobed the membrane for HA-SUMO-3 with a HA-specific antibody (Figure 7B). As expected, proteins with a wide variety of mobilities were observed due to the sumoylation of cellular proteins (Figure 7B). Nevertheless, the extract prepared from the cells coexpressing WT ERRα1 and HA-SUMO-3 clearly displayed a band migrating at the expected theoretical mobility (Figure 7B, lane 2). Again, this band was not seen in the cells expressing the ERRα1$_{K14R,K403R}$ mutant (Figure 7B, lane 4).

To further confirm that this novel band was, indeed, a sumoylated species of ERRα1, we cotransfected the cells with a plasmid encoding pcDNA3-FH-SENP1 (sentrin-specific protease 1). SENP-1 is a sumo-specific enzyme that removes SUMO from proteins (reviewed in *35*). We found that the presence of SENP-1 resulted in a large decrease in the accumulation of the higher mobility band (Figure 7C). Likewise, the novel band was also observed in the WT-, but not ERRα1$_{K14R,K403R}$, immunoprecipitate probed for HA-SUMO-3 with HA-specific antibody (Figure 7D, lane 2 vs lane 4). Thus, we conclude that ERRα1 can be sumoylated in MCF-7 cells.

We also performed in vitro sumoylation assays to determine if (i) ERRα1 could be sumoylated in vitro and (ii) ERRα1 was a target for SUMO-1 and SUMO-2 as well as SUMO-3. The assays were performed with ^{35}S-in vitro synthesized WT ERRα1, purified E1 activating enzyme SAE1/SAE2, and purified E2 conjugating enzyme Ubc9 in the presence or absence of the indicated SUMO. We found that ERRα1 could be sumoylated in vitro by SUMO-1, -2, and -3 (Figure 7E).

To determine whether Lys[14] or Lys[403] is the primary site of sumoylation on ERRα1, we also examined the effect on sumoylation of these amino acid residues individually and in combination. Cells were cotransfected and analyzed as above with plasmids expressing WT ERRα1, ERRα1$_{K14R}$, ERRα1$_{K403R}$, and ERRα1$_{K14R,K403R}$. Mutation of Lys[14] alone led to a significant, albeit incomplete, loss of sumoylation (Figure 7F, lane 4 vs lane 2). Mutation of Lys[403] alone led to an even greater, albeit still incomplete, loss of sumoylation (Figure 7F, lane 6 vs lane 2). Sumoylation was not observed at a detectable level above background with the double mutant (Figure 7F, lane 8). Thus, Lys[14] and Lys[403] are the two major amino acid residues on ERRα1 that are sumoylated in MCF-7 cells.

Sumoylation Represses ERRα1's Transcriptional Activity. To confirm that the effect on ERRα1's transcriptional activity observed with ERRα1$_{K14R}$ (Figure 6) was truly due to the

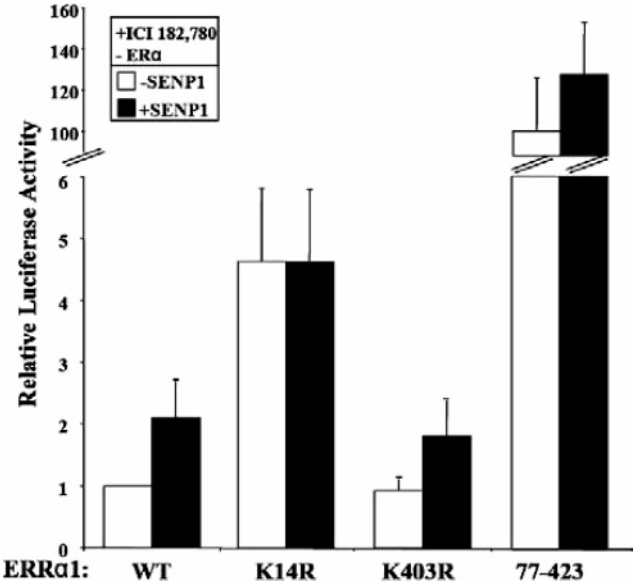

FIGURE 8: Effects of SENP1 on ERRα1 activity. MCF-7 cells were cotransfected in parallel with (i) an ERE-regulated or basal TATA-regulated luciferase reporter as an external control, (ii) the indicated ERRα1 expression plasmid, (iii) 0.1 μg of GRIP-1, and (iv) 0.2 μg of pcDNA SENP1 or its empty parental plasmid. Cells were incubated for 48 h in medium containing whole FBS in the presence of 10^{-7} ICI 182,780. After incubation, the cells were harvested and assayed for luciferase activity as described in the legend to Figure 2.

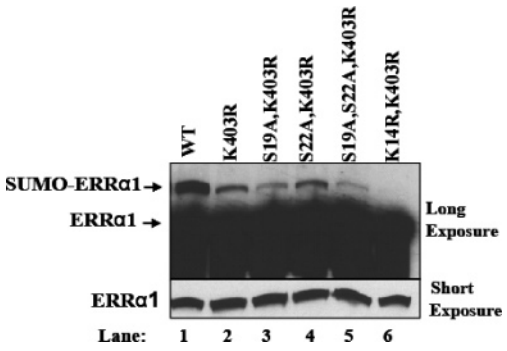

FIGURE 9: Phosphorylation at Ser^{19} promotes the sumoylation at Lys^{14}. MCF-7 cells were cotransfected with the indicated ERRα1 expression plasmid, pcDNA-6xmycUbc9, and pcDNA-HA-SUMO-3 or its parental vector. Cells were harvested 48 h after transfection, and whole-cell extracts were generated. Proteins from 5% of each whole-cell extract were processed as described in the legend to Figure 7D. Both a long and a short exposure of the blot are displayed to show relative quantities of ERRα1 and SUMO-ERRα1 among the mutants.

mutation preventing sumoylation, we also performed luciferase assays as described above in the absence or presence of pcDNA3-FH-SENP1. Thus, if sumoylation at Lys^{14} inhibits GRIP-1-mediated transcriptional activation by ERRα1, cotransfection of SENP1 should lead to an increase in the transcriptional activities of WT ERRα1 and $ERR\alpha1_{K403R}$ but have no effect on the transcriptional activity of $ERR\alpha1_{K14R}$. As predicted, overexpression of SENP1 resulted in a two-fold increase in the activity of WT ERRα1 and $ERR\alpha1_{K403R}$, yet failed to affect significantly the activity of either $ERR\alpha1_{K14R}$ or $ERR\alpha1_{77-423}$ (Figure 8). Thus, sumoylation at Lys^{14} has repressive effects on ERRα1's activity.

Phosphorylation of Ser^{19} Promotes Sumoylation at Lys^{14}. Last, we asked whether ERRα1 truly contains a PDSM such that phosphorylation at amino acid residues 19 and 22 effects sumoylation at Lys^{14}. To do so, we assayed for sumoylation of our phosphomutants in the presence of the K403R mutation so that we could observe the effects of sumoylation solely at Lys^{14}. Consistent with the presence of a PDSM, $ERR\alpha1_{S19A,K403R}$ showed a decrease in sumoylation as compared to $ERR\alpha1_{K403R}$ but still a readily detectable amount of sumoylation above that of $ERR\alpha1_{K14R,K403R}$ (Figure 9, lane 3 vs lanes 2 and 6). In agreement with the transcriptional phenotype (Figure 6), $ERR\alpha1_{S22A,K403R}$ showed no measurable loss of sumoylation (Figure 9 lane 4 vs lane 2), while $ERR\alpha1_{S19A,S22A,K403R}$ showed a level of sumoylation similar to that observed with $ERR\alpha1_{S19A,K403R}$ (Figure 9, lane 5 vs lane 3). Thus, we conclude that phosphorylation at Ser^{19} assists in promoting sumoylation at Lys^{14} but is not an absolute requirement for sumoylation to occur there.

DISCUSSION

In this report, we sought to gain insights into the mechanisms by which ERRα1 represses or activates ERE-dependent transcription. First, we showed that the amino-terminal domain of ERRα1 contributed to regulation of ERRα1's transcriptional activity in MCF-7 cells (Figure 2). Next, we identified the two major in vivo sites of phosphorylation of ERRα1 in MCF-7 cells to be located on amino acid residues 19 and 22 (Figures 3 and 4). Phosphorylation at Ser^{19} had repressive effects on ERRα1's transcriptional activities (Figure 5A), with the presence of a coactivator-binding site mutation in the AF-2 domain preventing GRIP-1-mediated transcriptional activation by the $ERR\alpha1_{S19A}$ mutant variant (Figure 5B). Mutation at Lys^{14}, a putative site of sumoylation, also led to an increase in GRIP-1-mediated transcriptional activation by ERRα1 (Figure 6A). We next identified Lys^{14} and Lys^{403} as the major sites of sumoylation of ERRα1 in MCF-7 cells (Figure 7), with sumoylation of Lys^{14} being a key regulator of ERRα1's transcriptional activity (Figures 6 and 8). Last, we showed that Ser^{19} and Lys^{14} are part of a PDSM in which phosphorylation of Ser^{19} can promote the sumoylation of Lys^{14} (Figure 9). Thus, we conclude that post-translational modifications within the amino-terminal domain of ERRα1 play significant roles in regulating ERRα1's transcriptional activity.

We found that inhibition of phosphorylation at Ser^{19} led to a two-fold increase in activation by ERRα1, while inhibition of sumoylation at Lys^{14} led to a 4−6-fold increase in activation (Figure 6A). This difference was consistent with our biochemical findings that phosphorylation at Ser^{19} enhanced sumoylation at Lys^{14} but was not absolutely necessary for this sumoylation to occur (Figure 9). Therefore, the partial increase in activation observed with $ERR\alpha_{S19A}$ was likely due to its partially decreasing sumoylation.

Recently, Yang et al. identified a negatively charged amino acid-dependent sumoylation motif (NDSM) (*37*). They found that acidic residues downstream of the core sumoylation consensus sequence, I/L/V-K-X-E, could enhance sumoylation, and phosphorylation could substitute for the acidic amino acid residues. From analysis of all validated, characterized sites of sumoylation, they concluded that this acidic patch of amino acids was most prevalent 3−6 amino acids downstream of the glutamic acid in the core sumoylation motif. Interestingly, Ser^{19} and Ser^{22} are located three and six amino acids, respectively, downstream of the glutamic

acid within ERRα1's Lys^{14} sumoylation site. Thus, phosphorylation at Ser^{22} likely also contributes to regulation of sumoylation at Lys^{14}, but the effects may not be as great and, therefore, not detectable here.

ERRα1's sumoylation at Lys^{403} is not regulated by a PDSM or NDSM. This is not surprising given that some sumoylated proteins do not even contain the core consensus sequence. It may explain why Lys^{403} appeared to be more sumoylated than Lys^{14} in MCF-7 cells (Figure 7D). Because the sumoylation of Lys^{14} is regulated, in part, by phosphorylation (Figures 6 and 9), its sumoylation may be more dynamic, thus resulting in less sumoylated Lys^{14} being present under steady-state conditions. No effect of $ERR\alpha1_{K403R}$ on transcriptional activity was observed here, showing the importance of the location of the sumoylation site. However, it remains quite plausible that sumoylation at Lys^{403} might effect ERRα1's transcriptional activity if the assays were performed with a different combination of promoter, coactivator, cells, or culture conditions. Another possibility is that sumoylation may be competing with another post-translational modification such as acetylation on Lys^{403} as seen with other proteins such as HIC1 (*38*). The Lys to Arg substitution mutants analyzed here inhibit acetylation as well as sumoylation. Thus, if inhibition of sumoylation enables an increase in acetylation of ERRα1, we would miss the possible effect on transcriptional activity with these mutants.

How does sumoylation at Lys^{14} affect ERRα1's transcriptional activity? The stabilities of WT ERRα1 and all of the sumo- and phosphomutant variants were similar (Figures 5 and 6). These mutations also did not affect ERRα1's stable binding as a homodimer to an ERE as measured by EMSAs (data not shown). Rather, inhibition of sumoylation at Lys^{14} increased ERRα1's responsiveness to GRIP-1 (Figure 6), a coactivator that is known to bind within the AF-2 domain of ERRα1 (*31*). This finding suggests that phosphorylation and sumoylation within the amino-terminal domain of ERRα1 regulate activity within the carboxy-terminal domain. The amino- and carboxy-terminal ends of other nuclear receptors such as androgen receptor can interact physically with each other to regulate transcription (reviewed in *39*). We hypothesize that regulation of ERRα1 may involve a similar interaction between its two terminal domains. Possibly, sumoylation at Lys^{14} regulates this interaction either directly or indirectly through an as-yet-unknown factor; removal of sumoylation alters this interaction, thereby enabling GRIP-1 to bind within the C-terminal domain. Alternatively, sumoylation may promote the binding of a corepressor to ERRα1. Another possibility is that SUMO, itself, possesses intrinsic repressive properties (reviewed in *35*).

The use of a sumo moiety to modulate transcriptional activity may be a common theme among at least some members of the nuclear superfamily. Iñiguez-Lluhí et al. (*21*) noted the presence of a sumoylation motif that functioned to repress its activity in the amino terminus of the glucocorticoid receptor. The presence of a SUMO moiety in the amino terminus of the progesterone receptor was found to block the activational response of the liganded C-terminal hormone-binding domain (*40*). Likewise, a site of sumoylation was identified in the N terminus of the androgen receptor, inhibiting its ligand-dependent activity (*41*). Lacking a small molecule ligand, ERRα1 is thought to be regulated in part by coactivators serving as its ligands (*42*). Thus, inhibition of coactivator response by sumoylation of the amino terminal end of ERRα1 is strikingly similar to the modulation of ligand-dependent progesterone and androgen receptor activities by sumoylation within their amino termini.

Whether ERRα1 represses or activates transcription depends upon a combination of promoter context, cell type, and cell-signaling pathways (*11*−*15*). For example, ERRα1's activities can be regulated by the ErbB2/EGFR signal pathways, with ERRα1 being phosphorylated by downstream kinases of these pathways such as PKC (*16*), Akt, and MAPK (*15*). Both ErbB2/EGFR and ERRα1 have been implicated in breast cancer (reviewed in *6*, *43*). Therefore, it would be of interest to determine whether the specific phosphorylation or sumoylation status of ERRα1 correlates with prognosis, therapeutic treatment, or patient outcome in this disease.

In summary, we have identified here functionally important in vivo sites of phosphorylation and sumoylation on ERRα1 in MCF-7 cells within the amino-terminal, ligand-independent A/B activation domain that contribute to ERRα1's transcriptional activity by inhibiting ERRα1's response to GRIP-1 within its carboxy-terminal AF-2 domain. This finding suggests mechanisms by which the activities of orphan receptors can be regulated in the absence of ligand binding. They may also open the way toward the development of clinically useful diagnostic agents and therapeutics.

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