Selective Mutations in Estrogen Receptor α D-domain Alters Nuclear Translocation and Non-estrogen Response Element Gene Regulatory Mechanisms*§

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The three main mechanisms of ERα action are: 1) nuclear, genomic, direct DNA binding, 2) nuclear, genomic, “tethered”-mediated, protein-protein interactions, and 3) non-nuclear, non-genomic, rapid action responses. Reports suggest the D-domain or hinge region of ERα plays an important role in mechanisms 1 and 2 above. Studies demonstrating the functionality of the ERα hinge region have resected the full D-domain; therefore, site directed mutations were made to attribute precise sequence functionality to this domain. This study focuses on the characterization and properties of three novel site directed ERα- D-domain mutants. The Hinge 1 (H1) ERα mutant has disrupted nuclear localization, can no longer perform tethered mediated responses and has lost interaction with c-Jun, but retains estrogen response element (ERE)-mediated functions as demonstrated by confocal microscopy, reporter assays, endogenous gene expression and co-immunoprecipitation. The H2 ERα mutant is non-nuclear, but translocates to the nucleus with estradiol (E2) treatment and maintains ERE-mediated functionality. The H2+NES ERα mutant does not maintain nuclear translocation with hormone binding, no longer activates ERE-target genes, functions in ERE- or tethered-mediated luciferase assays, but does retain the non-genomic, non-nuclear, rapid action response. These studies reveal the sequence(s) in the ERα hinge region that are involved in tethered-mediated actions as well as nuclear localization and attribute important functionality to this region of the receptor. In addition, the properties of these ERα mutants will allow future studies to further dissect and characterize the three main ERα mechanisms of action and determine the mechanistic role each action has in estrogen hormone regulation.

Many of the biological effects of estrogen are mediated through the estrogen receptors (ERs), ERα and ERβ, which belong to the nuclear receptor superfamily (1). Focusing on ligand-dependent activation, to date, there are three main mechanisms of action for ERα that include 1) nuclear, genomic, direct DNA binding, 2) nuclear, genomic, “tethered”-mediated protein-protein interactions, and 3) non-nuclear, non-genomic, rapid action responses (2–8). The classical ERα-mediated DNA binding responses are well established and are abrogated by specific disruption of two amino acid substitutions (E207A/G208A) in the DNA binding domain (10, 23). This ERα mutant only performs tethered and rapid action responses, as demonstrated by in vivo microarray of the mouse uterus showing that the tethered response accounts for ~25% of the WT transcripts (3). The functions of classical and rapid action-mediated ERα responses apart from the tethered-mediated ERα responses are not defined. Rapid action, non-nuclear ERα responses are attributed to the same ERα as the genomic responses (4–6, 12, 22, 24–27); however, it is challenging to separate the genomic ERα responses from the non-genomic ERα responses. Non-nuclear mechanisms are...
thought to involve activation of membrane-initiated kinase cascades. ERα does not have a transmembrane domain, but ERα interacts with caveolin-1 (6) and contains a palmitoylation sequence (28), which allows ERα to localize to the membrane. Rapid estrogen signaling via membrane-associated ERα leads to (a) MAPK, Akt, p21Cas, Raf, and PKC activation, (b) alterations of potassium channels, (c) increase in intracellular Ca2+ levels, and (d) release of nitric oxide and stimulation of prolactin secretion in various cell and tissue types (6, 29). The estrogen dendrimer complexes (EDCs) activate p44/42 MAPK (ERK1/2), Shc, and Src and are ineffective in stimulating endogenous estradiol target genes (30). Rapid action data obtained from the EDCs allow for ERα signaling from the membrane but, this method does not define non-nuclear actions alone as endogenous ERα is present and able to mediate genomic actions (5).

Previously, studies that have examined the hinge region of ERα have completely deleted the D-domain, rendering the receptor without the functionality of this domain (14, 31, 32). These studies attributed to the understanding of the functionality of the D-domain of ERα, but the exact sequences in the hinge region of ERα critical for nuclear localization and interaction with c-Jun are not identified. Functionality of cytoplasmic/membrane only ERα or functionality of ERα apart from tethered-mediated activity have not been described. Therefore, with previous literature suggesting that the D-domain is involved in these functions of ERα, we sought to create specific ERα mutants to define these actions. Mutations in the hinge region of ERα were generated by site directed mutagenesis rather than deletion mutations. Hinge mutants H1 ERα, H2 ERα, and H2+NES ERα have unique properties that block ERα tethered-mediated effects, block nuclear localization without ligand, and block liganded nuclear genomic-mediated actions while maintaining non-nuclear, rapid action responses, respectively. These ERα mutants define sequence(s) in the ERα D-domain responsible for actions involving nuclear localization and gene selective activation and demonstrate that the D-domain of ERα is critical for not only receptor nuclear localization but also in selective transcriptional regulation.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—17β-Estradiol (E2) was purchased from Sigma-Aldrich and ICI 182,780 was from Tocris Biosciences (Ellisville, MO). Anti-ER antibodies were from Cell Signaling Technology (Danvers, MA). pho-Src (Y527) (#2105), non-phospho-Src (Y527) (#2107) phospho-Akt (S473) (#4060), Akt (#9272), phospho-Src (Y416) (#2101), non-phospho-Src (Y416) (#2102), phospho-Src (Y527) (#2105) and non-phospho-Src (Y527) (#2107) antibodies were from Cell Signaling Technology (Danvers, MA).

Plasmids and Constructs for ERα Mutants—pcDNA vector was purchased from Invitrogen (Carlsbad, CA) and pAP-1-Luc (AP-1 Luc) was purchased from Stratagene (La Jolla, CA). The following were gifts: pcDNA3/SRC-2 (D. McDonnell), pRSV/c-Jun (M. Karin), Sp-1 reporter (L. Jameson). pGL3/3xERE Luc (3x ERE Luc) reporter and pcDNA/mouse ERα have been described previously (33, 34). pcDNA/mouse (m) Hinge 1 (H1)-ERα was introduced by creating overlapping oligos using ERα unique restriction enzyme sites BsmI and XbaI. A 306-bp fragment created from a 156-bp forward primer and a 200-bp reverse primer introducing R267A, K270A, K272A, K273A, and K275A mutations was cloned and ligated back into pcDNA/mERα via directional cloning into the BsmI and XbaI sites. The pcDNA/Hinge 2 (H2)-mERα mutant was also generated using a 306-bp fragment created from a 156-bp forward primer and a 199-bp reverse primer introducing R260A, K261A, R263A, R264A, R267A, K270A, K272A, R273A, and K275 mutations was cloned and ligated back into pcDNA/mERα via directional cloning into the BsmI and XbaI sites. The pcDNA/H2+NES-mERα mutant was generated from pcDNA/H2-mERα to introduce the nuclear export signal (NES) by using QuikChange Lightning (Stratagene) to introduce R273L and D274L thus creating the sequence shown in Fig. 1A. The pcDNA/NES-mERα was generated from pcDNA/mERα by using QuikChange Lightning to introduce R273L and D274L. To tag the ERα mutants with GFP for cellular localization analysis, each ERα construct was subcloned from the pcDNA3/vector into pEGFP-C1 (Clontech, Mountain View, CA). pcDNA/mERα was opened with Sphi and blunted with Klenow (New England Biolabs, Ipswich, MA) and then cut with EcoRI. The mERα and mERα mutants were ligated into the EcoRI and Smal sites of pEGFP-C1.

Cell Lines and Tissue Culture—Human kidney cell line 293F was purchased from Invitrogen, and human cervical epithelial cell line HeLa and human kidney cell line HEK293T/17 were purchased from ATCC (Manassas, VA). Ishikawa, human uterine epithelial cells, have been described previously (35). 293F were maintained in phenol-red free DMEM (Gibco-Invitrogen, #21063) supplemented with 10% FBS (BenchMark, Gemini Bio-Products, West Sacramento, CA), and HeLa Ishikawa cells were maintained in phenol-red free DMEM/F12 (Gibco-Invitrogen, #11039) supplemented with 10% FBS.

Production of Lentivirus and Stable Cell Lines—All lentivirus was packaged in HEK293T/17 cells according to published protocols (36). Briefly, 293T cells were transiently transfected with pMD2G, psPAX2, and pDEST673 carrying the neomycin resistance gene and the desired ERα mutant using Lipofectamine 2000. Supernatant was collected 48 h post transfection and concentrated by centrifugation at 50,000 × g for 2 h. Pellets were resuspended in PBS and used for infection. Titers were determined using quantitative PCR to measure the number of lentiviral particles integrated into the host genome. MOI ranging from ~180 to 25 were used for infection of Ishikawa cells. After 3 days of infection, cells selected with Geneticin (1.2 mg/ml, Invitrogen, #11811-031) and a stably pooled population of cells was obtained after 2 weeks. Stable integration of ERα was verified by Western blot for ERα.

Transient Transfection and Luciferase Assay—Cells were seeded in 24 well plates overnight. A total of 0.5 μg of DNA, including 0.2 μg of expression plasmids (pcDNA/vector, pcDNA/ERα, pcDNA/H1-ERα, pcDNA/H2+NES-ERα, or pcDNA/NES-ERα) 0.2 μg of reporter plasmids (3xERE Luc or AP-1 Luc) and 0.1 μg of pRLTK plasmids, were transfected overnight using the Effectene transfection reagent (Qiagen,
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Valencia, CA) according to the manufacturer’s protocol. Cells were starved with 10% HyClone Charcoal/Dextran-stripped FBS (sFBS) (Thermo Scientific, Waltham, MA) for 8 h and then were treated as described in the figure legends. For luciferase experiments co-expressing c-Jun, cells were plated and transfected as above with a total of 0.5 μg of DNA, including 0.2 μg of reporter plasmid, 0.1 μg of ERα expression plasmids and/or 0.1 μg of pRSV/c-Jun and 0.1 μg of pRLTK. For experiments co-expressing SRC-2, cells were plated and transfected as above with a total of 0.75 μg of DNA, including 0.2 μg of reporter plasmid, 0.1 μg of ERα expression plasmid, 0.4 μg of pcDNA3/SRC-2, and 0.05 μg of pRLTK. Luciferase assays were performed using the Dual-Luciferase Reporter Activity System (Promega, Madison, WI) according to the manufacturer’s protocol. Data are from three independent experiments.

Confocal Microscopy—HeLa cells were grown overnight on Lab-Tek 2 well chamber slides (NUNC, Rochester, NY), transfected with 0.5 μg of pEGFP-ERα expression plasmids as described above. Cells were starved with 10% sFBS for 8 h and then were treated as described in the figures and figure legend. The cells were then fixed with 4% paraformaldehyde for 1 h. Cells were washed with PBS and coverslipped with ProLong Gold Anti-Fade reagent with DAPI (Invitrogen, #P-36931). The cellular localization of ERα was visualized by confocal microscopy (Zeiss LSM-510 equipped with an argon-krypton laser) using a 40 × 1.2 objective lens.

For the Western blot, whole cell lysates were obtained from cells transiently transfected with ERα expression plasmids using RIPA buffer (50 mM Tris-Cl (pH 7.6), 1 mM EDTA (pH 8.0), 0.5 mM EGTA (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protease inhibitor mixture (Sigma)) and 10 μg of whole cell lysate was run on a 10% Tris-glycine gel, transferred to nitrocellulose and the ERα was detected by anti-ERα (MC-20) and GAPDH by anti-GAPDH using the ECL Plus Detection System (Amersham Biosciences™, GE Healthcare Biosciences, Corp., Piscataway, NJ).

Co-immunoprecipitation (co-IP)—293F cells were plated in 10-cm dishes overnight. Cells were transfected overnight as above with 2 μg of pcDNA/ERα plasmid (WT and H1 mutant) and/or 3 μg of pRSV/c-Jun. Cells were treated for 36 h with vehicle or 10 mM E2. Cell lysates were prepared in 400 μl of RIPA buffer. HIP buffer (50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1% Triton X-100 and protease inhibitor mixture) was added to the cell lysate to a final volume of 4 ml. The cell lysates were pre-clearced with protein A-Sepharose (Amerham, CL-4B, 17-0780-01) for 1 h at 4°C with rocking. Protein lysates (1 ml) were immunoprecipitated by using 5 μg of anti-ERα (H-184), anti-c-Jun, rabbit IgG, and mouse IgG overnight at 4°C with rocking. The protein antibody complexes were bound to protein A-Sepharose, suspended in the lysis buffer, for 4 h at 4°C with rocking. The bound complexes were washed 6 times with HIP buffer, precipitated, and boiled in SDS sample buffer. The immunoprecipitated products were then resolved with the 10% Tris-glycine gel system. Proteins were transferred to nitrocellulose and the presence of ERα or c-Jun in the precipitants was detected by anti-ERα (MC-20) or c-Jun. The immunoreactive products were detected by the ECL Plus Detection System.

RNA Extraction and Real-time PCR—Total RNA was extracted by using TRizol Reagent (Invitrogen) according to the manufacturer’s protocol. First-strand cDNA synthesis was performed using superscript reverse transcriptase according to the manufacturer’s protocol (Invitrogen). The mRNA levels of progesterone receptor (PR), pS2, and p21 were measured using SYBR green assays (Applied Biosystems). The sequences of primers used in real-time PCR were as follows: for human PR (NM_000926.4): the forward primer 5’-GAGTTGGGAG-GGCGCATAT-3’, reverse primer 5’-GCAGTCGGCTGTCCT-TTTCT-3’, for human pS2 (NM_002252.3): the forward primer 5’-GCCCTCCCAGTCTGCAAATA-3’, reverse primer 5’-CTGGAGGAGCGCTGAGTTA-3’, for human p21 (NM_078467.1): the forward primer 5’-CTGTACTGCTGT-TGTACCC-3’, reverse primer 5’-GCCGTTGAGGAGCTGAATC-3’ and then normalized with respect to the control group. Quantification was performed according to the mathematical model described by Pfaffl (37) and as previously described (33). The experiments were repeated three times and results are presented as fold increase ± S.D.

MAPK Analysis—Cells were seeded in 60-mm dishes, cultured overnight in phenol red-free DMEM:F12 medium with 10% FBS, and transfected with 2 μg of ERα expression plasmids as indicated in the figure legends for 8 h following the Effectene manufacturer’s protocol. The transfected cells were then starved in phenol red-free DMEM:F12 with 0.1% sFBS for 3 days. Ishikawa stable cell lines were seeded and starved as above. Cells were treated with 100 nM E2, for 0, 3, 5, 10 min. Cells were placed on ice, washed with cold PBS, and lysed in ice-cold lysis buffer (1% Igepal (Sigma #56741), 0.5% sodium deoxycholate, 0.1% SDS, 1× complete mini-tab (Roche 11-836-170-10), 10 mM sodium fluoride, and 1 mM sodium orthovanadate), for 30 min followed by sonication with a probe sonicator for 15 s on ice (setting 6 on 60 Sonic Dismembrator from Fisher Scientific). The supernatant (2 μg) was used for Western blot analysis as described above.

Statistical Analysis—One-way ANOVA with Tukey’s post-test was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA. Two-way ANOVA with Bonferroni post-test was also performed using GraphPad Prism version 5.00.

RESULTS

Selective Mutations in the D-domain of ERα Disrupt Nuclear Localization—The D-domain of ERα is important for nuclear localization and interaction via the tethered mechanism (10, 20, 21). Computational analysis of ERα by LOCtree and Motif Scan suggested putative nuclear localization signals in the C-domain and D-domain (amino acids 234–240 and 270–275; amino acids 247–263 and 260–276, respectively). Amino acids 234–
240 are located in the C-domain/DNA binding domain of ERα. Amino acids 257–276 are located in the D-domain/hinge region and analysis of the suggested nuclear localization signals in the hinge region, revealed two putative bipartite nuclear localization sequences (NLS—K-(K/R)-X-(K/R)). The putative nuclear localization sequences are 100% conserved between mouse and human ERα (Fig. 1A), and in addition, this area is not conserved between ERα and ERβ as seen with alignment analysis. To further characterize the hinge region and to precisely define the sequences involved in ERα nuclear localization and ERα tethered-mediated responses, site-directed mutagenesis was performed in this area of the hinge region of ERα at the sites shown in Fig. 1B.

Confocal microscopy was used to demonstrate ERα localization by visualizing enhanced GFP-tagged ERα and mutant ERα proteins in transiently transfected HeLa cells (Fig. 1C). WT ERα is predominately nuclear in the absence or presence of ligand, E2. Examination of the H1 mutant, where part of the bipartite NLS is mutated to alanine, demonstrated a disruption of nuclear localization in the absence of ligand; however, in the presence of E2, this mutant translocated to the nucleus. The H2 ERα mutant, having the full bipartite NLS mutated to alanine, is non-nuclear in the absence of ligand, but in the presence of E2, translocation to the nucleus is observed. To force exclusion of the ERα from the nucleus the NES ERα mutant was engineered to have a nuclear export signal (NES—LXXXLXXLXL (38)) in the same area of the hinge region as the putative NLS. The NES ERα mutant translocates to the nucleus in the absence and presence of ligand as is seen with WT ERα demonstrating the strength of the ERα nuclear localization signal(s) and the nucleophilic nature of ERα. We then created the H2+NES ERα mutant which has the full bipartite NLS mutated to alanine with the addition of a nuclear export signal within the putative NLS region. The H2+NES ERα was not detected in the nucleus under normal conditions (Fig. 1C). Repeating the ERα localization experiment in the presence of leptomycin B (LMB), a nuclear export inhibitor, the H2+NES ERα was detected in the nucleus (supplemental Fig. S1). This suggests that the H2+NES ERα, presumably due to the NES, is rapidly shuttled or pumped out of the nucleus. Additionally, mutating the suggested nuclear localization sequence in the C-domain/DNA binding domain does not disrupt nuclear localization (data not shown). A Western blot for ERα protein and all the mutants is shown in Fig. 1D. The H1, H2, H2+NES, and NES ERα mutant constructs were utilized in this study to assess receptor functionality.

H1 ERα Mutant Loses Tethered-mediated Responses but Maintains ERE-mediated Activation—To analyze nuclear responses, the functionality of the H1 ERα mutant by promoter activation was examined. Luciferase assays were performed...
with reporters to observe classical (3× ERE Luc) and tethered (AP-1 Luc)-mediated reporter activation of the ERα mutant. Reporter activation of the ERE Luc is seen with both WT ERα and the H1 ERα mutant when stimulated with E2 and the response is blocked by the ERα antagonist, ICI-182,780 (Fig. 2A). The AP-1 reporter, as previously published is activated by ICI-182,780 (7, 10, 39), was used to examine the tethering ability of the H1 ERα mutant. Activation of this reporter is abrogated in the H1 ERα mutant (Fig. 2A). Ishikawa, uterine epithelial cells, were stably infected with empty vector, WT ERα, or H1 ERα (supplemental Fig. S2). To validate the reporter assay data, progesterone receptor (PR) (33), a classical ERE-mediated response, and p21 (3), a tethered-mediated response, were examined. WT and the H1 ERα mutant activate via the classical ERE-mediated response; however, the H1 ERα mutant is no longer activated via the tethered-mediated response (Fig. 2B). In addition, the same loss in tethered activity is observed with the H1 mutant using the Sp1 reporter containing the Sp1 response element (data not shown). These results suggest that the H1 ERα mutant retains its ability to translocate to the nucleus and bind to DNA in the presence of E2, but has lost its ability to interact with the necessary tethering factors to activate via the AP-1 and Sp1 response elements.

H1 ERα Does Not Form a Protein-Protein Complex with c-Jun—ERα and the AP-1 family member c-Jun directly interact in the hinge region of ERα and this interaction is responsible for AP-1-mediated tethered responses (21). To investigate if the AP-1 tethered response could be rescued by co-expression of c-Jun, cells were co-transfected with the ERα plus or minus c-Jun expression plasmids. As evidenced by the AP-1 luciferase assay, enhancement of activity was only observed in cells co-transfected with WT ERα and c-Jun. Co-expression of c-Jun did not rescue this response with the H1 ERα mutant (Fig. 3A). H1 ERα was used to determine if the AP-1-mediated inactivity was due to a loss in protein-protein interaction of H1 ERα and c-Jun. Cells co-transfected with ERα and c-Jun exhibit protein-protein interaction as demonstrated by both ERα and c-Jun immunoprecipitation (Fig. 3B). This interaction is stronger under E2 hormone binding conditions; therefore, experiments with the H1 ERα mutant were done with E2 treatment. Co-immunoprecipitation was performed on cells co-transfected with WT ERα + c-Jun or H1 ERα + c-Jun expression constructs. Immunoprecipitation for ERα and c-Jun did not co-immunoprecipitate c-Jun or ERα, respectively, in cells co-transfected with the H1 ERα mutant (Fig. 3C). These data suggest that the loss of tethered-mediated activity is due to a loss in the ability of ERα to form the proper protein-protein complex involving this specific sequence in the D-domain of ERα necessary for mediating this particular mechanism of ERα action.

Co-activation of ERα by the steroid receptor co-activators (SRCs) are known to occur by protein-protein interactions in the E/F-domain of ERα (40). Therefore, to determine if the H1 ERα mutant was still able to interact with co-activators and the

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**FIGURE 2. Site-directed mutagenesis of R/K residues to A in the hinge region of ERα disrupts tethered-mediated promoter activation.** A, luciferase activity with the ERE-luciferase reporter or the AP-1-luciferase reporter and the ERα expression plasmids. For ERE-luciferase activity, transiently transfected 293F cells were treated with vehicle (0.01% DMSO), E2 (10 nM), ICI-182,780 (1 μM), or E2 + ICI for 18 h. For AP-1-luciferase activity, transiently transfected 293F cells were treated with vehicle (0.01% DMSO) and ICI-182,780 (100 nM) for 18 h. Transfection efficiency was normalized by Renilla luciferase. Data shown are representative of three experiments. *p < 0.05, one way ANOVA with Tukey’s Multi Comparison post-test. B, total RNA was isolated from Ishikawa cells stably expressing empty, WT ERα, or H1 ERα mutant. Cells were treated with vehicle (0.01% DMSO) or E2 (10 nM) for 4 or 24 h. PR (24 h) and p21 (4 h) transcripts were quantified by real time-PCR as described under “Experimental Procedures” and were calculated relative to vehicle. *p < 0.05-two-way ANOVA with Bonferroni post-test.
tethered-mediated response was specific to this sequence of the hinge region, ERE- and AP-1-mediated luciferase assays with SRC2 co-expression were performed. Co-expression of SRC-2 stimulates the ERE-mediated response of WT and the H1 ERα mutant. However, the H1 ERα mutants still remains inactive in the AP-1-mediated luciferase assay (Fig. 4, A and B). Similar results were obtained with SRC-1 and SRC-3 co-expression (data not shown). These data suggest that the H1 ERα mutant still retains the ability to interact with co-activators that are necessary for the ERE-mediated responses while lacking the tethered-mediated function.

Non-nuclear Cytoplasmic Signaling of H2+NES ERα Is Maintained while Nuclear Genomic Responses Are Lost—The H2 ERα mutant, with the full bipartite NLS mutated to alanine, is cytoplasmic with vehicle conditions, but translocates to the nucleus with E2 treatment. To prevent the nucleophilic ERα from maintaining nuclear localization, and keeping the focus on the D-domain, a nuclear export signal (NES; LXXXLXX-LXL) was added (Fig. 1, B and C). For the study of non-nuclear, rapid action only events, the ERα must not perform genomic-mediated responses (ERE and tethered). Promoter activation of the H2, NES and H2+NES ERα mutants were examined using the ERE and AP-1 luciferase reporters. In the ERE-mediated assay, the H2 and the NES ERα mutants responses were comparable to that of the WT ERα response (Fig. 5A), but the response is abrogates in the H2+NES ERα mutant. As expected, the H2 and the H2+NES ERα mutants no longer activated the AP-1-mediated reporter while the NES ERα mutant maintained activity (Fig. 5A). To further examine the ERE-mediated nuclear genomic function of these mutants, endogenous ERE-mediated target gene expression was validated in 293F cells transiently transfected with WT ERα, H2 ERα, NES ERα mutant, and H2+NES ERα mutant expression plasmids. Endogenous gene expression of PR and trefoil factor 1 (pS2) (ERE-mediated target genes (33)) were examined by real-time RT-PCR (Fig. 5B). PR and pS2 are induced by E2 in the WT ERα, H2 ERα mutants and the NES ERα mutant transfected cells; however, the H2+NES ERα mutant is unable to activate nuclear target gene expression. In addition, we confirmed the ERE-mediated target gene expression in stably infected Ishikawa cells (WT and H2+NES ERα) by examining PR expression levels (supplemental Fig. S3A). The lack of ERE- and tethered-mediated responses in combination with the nuclear localization results with the H2+NES ERα mutant further sug-
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A

FIGURE 5. H2+NES ERα loses nuclear genomic functions. A, luciferase activity with the ERE-luciferase reporter or the AP-1-luciferase reporter and the ERα expression plasmids. For ERE-luciferase activity, transiently transfected 293F cells were treated with vehicle (0.01% DMSO), E2 (10 nM), ICI-182,780 (1 μM), or E2 + ICI for 18 h. For AP-1-luciferase activity, transiently transfected 293F cells were treated with vehicle (0.01% DMSO) and ICI-182,780 (100 nM) for 18 h. lntersection efficiency was normalized by Renilla luciferase. *, p < 0.05, one way ANOVA with Tukey’s Multiple Comparison post-test. B, total RNA was isolated from 293F cells transiently transfected with WT ERα, H2 ERα, NES ERα, or H2+NES ERα mutants and treated with vehicle (0.01% DMSO) or E2 (10 nM) for 24 h. PR and pS2 transcripts were quantified by real time-PCR as described under “Experimenal Procedures” and were calculated relative to vehicle. WT ERα, H2 ERα, NES ERα, and H2+NES ERα were tested versus their vehicle control by two-way ANOVA with Bonferroni post-test (*, p < 0.05).

B

suggests that the loss in activity is consistent with the inability of the mutant receptor to maintain nuclear translocation.

To establish that a potential loss of genomic function was not due to a loss in DNA binding ability, an ERE transcription factor ELISA assay was performed. The H2+NES ERα binds DNA comparable to WT ERα suggesting that any loss of genomic function is not due to lack of DNA binding (supplemental Fig. S3B).

To verify the functionality of the H2+NES ERα mutant, the non-nuclear, non-genomic, rapid action response of this mutant was examined. ERα deficient, HeLa cells were transiently transfected with empty vector, WT ERα, or the H2+NES ERα mutant constructs. Concomitantly, low expressing ERα Ishikawa cells, were stably transfected with the ERα constructs (supplemental Fig. S2). Both cells were starved for 60 to 70 h to decrease basal phospho-p44/42 MAPK as described under “Experimental Procedures.” To stimulate the rapid action response, the transfected HeLa cells were treated for 0, 3, 5, and 10 min with E2 (100 nM) and the stable Ishikawa cell lines were treated for 0, 5, and 10 min with E2. An increase in phospho-p44/42 MAPK was observed in WT ERα and H2+NES ERα mutant expressing cells after 3 and 5 min of E2 stimulation (Fig. 6). These data show that the H2+NES ERα mutant, unable to maintain nuclear translocation, retains the properties for mediating non-genomic rapid action responses comparable to WT ERα.

DISCUSSION

Traditionally, the D-domain ERα mutations used to demonstrate the ability to interact with proteins in a tethered-mediated fashion and NLS functionality have been with mutations of ERα containing complete deletions of the described D-domain or the suspected NLS (13, 14, 19–22). Complete deletion of this domain would, presumably, result in dramatic alterations in receptor protein structure. To address the portion of the ERα sequence in the hinge region responsible for gene regulation via the tethered mechanism and nuclear localization, we chose to make specific site-directed changes to the ERα hinge region as opposed to domain deletions.

Computational analysis (LOCtree and Motif Scan) of ERα revealed putative nuclear localization signals in the C- and D-domains (AA 234–240 and 270–275; 247–263 and 260–276, respectively). The hinge region is the least conserved region between mouse and human ERα, but the nuclear localization sequences are 100% conserved from mouse to human as shown in Fig. 1A. These predicted nuclear localization signals correlated to those suggested by Ponglikitmongkol et al. (32) and Ylikomi et al. (14). The ERα deletions made by Ylikomi et al. disrupted nuclear localization, but did not completely exclude ERα from the nucleus. To define the precise sequence responsible for nuclear localization in ERα, we mutated the R/K residues in the predicted area to A. When R/K residues from AA 234 to 256 in C-domain/exon 4 were mutated to A (8 R/K to A substitutions in the 2nd zinc finger of the C-domain), nuclear localization was not disrupted (data not shown). We then chose to focus on the basic amino acids in the D-domain/exon 5 from 260 to 281 that suggested a bipartite nuclear localization signal. When half (5 R/K to A substitutions- H1 ERα mutant) of the bipartite nuclear localization signal was mutated, ERα was observed to be more cytoplasmic under vehicle conditions than WT ERα, but fully translocated to the nucleus when treated
with E2 (Fig. 1C). Mutation of the full putative bipartite nuclear localization signal (9 R/K to A substitutions—H2 ERα mutant) was required for nuclear exclusion in the absence of ligand, but with E2 treatment, partially translocated (C=N) to the nucleus.

To counter the ligand-dependent nuclear localization of the H2 ERα mutant and to keep experimental focus on the functionality of the hinge region, a NES flanking the mutated putative nuclear localization signal sequence in the hinge region was incorporated. H2 + NES ERα is blocked from maintaining nuclear localization even with hormone treatment, while NES ERα harboring only the addition of a nuclear export signal, is not excluded from the nucleus (Fig. 1C). The H2 ERα mutant must be counteracted by a nuclear export signal to keep the nucleocentric ERα out of the nucleus. The glucocorticoid receptor (GR) has a nuclear retention signal that overlaps the nuclear localization function and is necessary for ligand bound transcriptional activation (41). A similar nuclear retention signal or event for ERα is possible and the cytoplasmic versus nuclear distribution of the H1 and H2 ERα mutants suggest that this region of ERα may be responsible for ligand-dependent nuclear retention of ERα. Further studies are necessary to confirm whether nuclear retention of ERα is through phosphorylation or protein-protein-mediated interactions as others have noted (42, 43).

Functional analysis of H1 and H2 ERα revealed direct DNA binding activities were unaffected. However, the AP-1 mediated luciferase response was lost with H1 and H2 ERα (Figs. 2B and 5B). H1 ERα, having less amino acid substitutions than H2 ERα and still maintaining the abrogation in AP-1-mediated activity, demonstrated that this loss in activity is likely due to loss of direct protein-protein interaction between H1 ERα and c-Jun (Fig. 3). The loss in AP-1-mediated activity due to a loss in protein-protein interactions (21) is not remarkable, but it is remarkable that the sequence mutated in ERα that abrogates this interaction (RMLKHKQR → AMLAHAAQA) is a conserved nuclear localization consensus sequence. The H1 ERα Ishikawa stable cells demonstrated the loss in activity of an E2 AP-1 responsive gene, p21. Great promoter redundancy and cell type specificity is seen with AP-1 and Sp1 target genes (44); therefore, conducting microarray analysis on the H1 ERα stable cell line to examine gene expression changes over a time course may uncover unknown E2 ERα tethered-mediated gene responses.

The hinge region of nuclear receptors, initially thought to allow flexibility of the receptor, is demonstrating functionality in transactivation in other nuclear receptors as well. This region is regulated by postranslational modifications such as methylation (45, 46), acetylation (47), and sumoylation (48). The hinge region of androgen receptor (AR) and vitamin D receptor (VDR) regulates nuclear localization, DNA binding, and plays a role in coactivator recruitment and N/C-terminal interactions (17, 49). The GR hinge region interacts with HEXIM1 and Bag-1 M which mediates glucocorticoid-mediated transcriptional repression of biological responses (17, 50, 51). The hinge region of ERα is post translationally modified; the sites important in human ERα for methylation and acetylation are Lys-302 and Lys-303 which correspond to Lys-306 and Lys-307 in mouse ERα (52, 53). Sumoylation sites that are involved in the regulation of ERα transcriptional activity are human ERα sites Lys-299,302 and Arg-303 (mouse ERα K303, 306 and R307) with K266 and R268 (mouse ERα Lys-270 and Arg-272; located in our H1 ERα mutant) demonstrating no change in transcriptional activity (48). Kim et al. (54) show mutant K266Q/R268Q (mouse ERα K270/R272) when acetylated, increases ERα’s DNA binding interaction with p300. In our experiments, we saw no change in DNA binding of the H1 ERα mutant, but this is likely due to the constitutive activity of the K266Q/R268Q mutant.

In contrast to H1 and H2 ERα, H2 + NES ERα demonstrates a loss in the ability to perform nuclear genomic responses involved in the classical and tethered mediated actions of ERα while maintaining the ability to bind DNA and perform non-nuclear, non-genomic, rapid action mediated phosphorylation of p44/42 MAPK (Fig. 5). This is in contrast to Zhang et al. (24) who do not see phospho-p44/42 MAPK with WT ERα and their NLS deletion mutant, HE241G, (deletion of amino acids 256–303 of human ERα, but do see p44/42 MAPK phosphorylation with their NLS myristoylation tagged mutant HE241G-mem. Chambliss et al. (22) demonstrate that with deletion of the NLS-hinge (amino acids 250–274 of human ERα) eNOS is no longer regulated via the non-genomic p44/42 MAPK response. The discrepancies presented here may be attributed to the deletion of 47 or 24 amino acids in these mutated ERα constructs, respectively, that disrupts proper protein folding compared with our point mutation approach. Many of the rapid action studies which do not use endogenous ERα, but require transfection of ERα into the cells, focus on p44/42 MAPK activity. We find that in HeLa and Ishikawa ERα transfected cells, there is no change in activity of Akt (Ser-473), Src (Tyr-416 or Tyr-527), or GSK3β (Ser-9) with E2 treatment. This finding highlights the sensitivity and the cell type specificity of the E2-mediated rapid action responses.

Localization and function of ERα at the plasma membrane requires Ser-522 of human ERα (26). A membrane-targeted ERα variant created by attaching myristoylation and palmitoylation sequences to the N and C terminus, respectively, of ERα missing the putative nuclear localization signals (human amino acids 256–303) demonstrate membrane localization in the absence of estradiol and this mutant failed to regulate endogenous estradiol-responsive genes (31). A MOER (membrane-only estrogen receptor α) model has been generated, but in contrast to our H2 + NES ERα mutant, the ERα receptor in this mouse contains only the E-domain with the addition of multiple palmitoylation sites to direct this form of the receptor to the cell membrane (55). Our H2 + NES mutant, without the added myristoylation and palmitoylation tags, but retaining the natural sites for this activity, exhibits a similar phenotype by no longer retaining the ability to activate E2-mediated target genes, activate via ERα- or AP-1-driven reporter assays, is cytoplasmic without E2, and demonstrates rapid action responses via phospho-p44/42 MAPK. Additionally, H2 + NES ERα is unique because the backbone spacing of ERα is retained and likely preserves ERα folding more adequately than deletion mutants.

The hinge mutants generated here are important tools to tease apart the three modes of action of ERα from one another.
Selective ERα D-domain Mutations

This functionality compliments published and ongoing studies in our laboratory, that utilize knock-in mouse models to examine the physiological relevance of the various functions of ERα (2). The models developed to date nicely attribute phenotypic changes to different functional regions of ERα and suggest that the mutations characterized here, will also demonstrate phenotypic changes important to biochemical pathways and estrogen biology.

In conclusion, the ERα mutants studied here are specific D-domain site directed mutants that demonstrate unique properties. We find as others that ERα harbors multiple nuclear localization sequences that renders this receptor in the absence of hormone to mainly be localized to the nucleus, and this is in contrast to ERβ, which contains only one potential putative NLS and is very often localized in the cytoplasm and translocates to the nucleus slowly over a 6–12 h time period (56). Our experiments demonstrate the strength and utility of ERα nuclear localization signals and genomic mediated activities.

Mutation of half of the bipartite NLS (H1 ERα) only slightly disrupts nuclear localization but abrogates the tethered genomic response while maintaining ERE-genomic responses. Mutating the full bipartite NLS blocks nuclear translocation without ligand, but the addition of hormone translocates this receptor to the nucleus where it is able to activate ERE-mediated responses (H2 ERα). The addition of a nuclear export signal in the putative NLS region of ERα does not disrupt localization or activity of the NES ERα mutant. A nuclear export signal must be added in addition to mutation of the nuclear localization sequence(s) to exclude ERs from the nucleus and render this form of the receptor unable to perform genomic mediated responses (H2+NES ERα). This study assigns functionality to specific sequences in ERα D-domain involved in nuclear localization and tethered-mediated responses, and provides new avenues to fully dissect and use these ERα mutants to understand the varied functionality of estrogen and ERα in different tissues and to create therapeutic drug targets which could regulate a precise activity of ERα apart from the other modes of action.

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REFERENCES

1. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) Cell 83, 835–839
2. Hewitt, S. C., Li, Y., Li, L., and Korach, K. S. (2010) J. Biol. Chem. 285, 2676–2685
3. Hewitt, S. C., O’Brien, J. E., Jameson, J. L., Kissing, G. E., and Korach, K. S. (2009) Mol. Endocrinol. 23, 2111–2116
4. McDevitt, M. A., Gidewell-Kenney, C., Jimenez, M. A., Ahearn, P. C., Weiss, J., Jameson, J. L., and Levine, J. E. (2008) Mol. Cell. Endocrinol. 290, 24–30
5. Madak-Erdogan, Z., Kieser, K. J., Kim, S. H., Komm, B., Katzenellenbogen, J. A., and Katzenellenbogen, B. S. (2008) Mol. Endocrinol. 22, 2116–2127
6. Song, R. X., Zhang, Z., and Santen, R. J. (2005) Trends Endocrinol. Metab. 16, 347–353
7. Kushner, P. I., Agard, D. A., Greene, G. L., Scanlan, T. S., Shiu, A. K., Uht, R. M., and Webb, P. (2000) J. Steroid Biochem. Mol. Biol. 74, 311–317
8. Webb, P., Nguyen, P., Valentine, C., Lopez, G. N., Kwok, G. W., McNenmy, E., Katzenellenbogen, B. S., Enmark, E., Gustafsson, J. A., Nilsson, S., and Kushner, P. J. (1999) Mol. Endocrinol. 13, 1672–1685
9. Glass, C. K. (1994) Endocrine Rev. 15, 391–407
10. Jakacka, M., Ito, M., Weiss, J., Chien, P. Y., Gehm, B. D., and Jameson, J. L. (2001) J. Biol. Chem. 276, 13156–13162
11. Safe, S. (2001) Vitamins Hormones 62, 231–252
12. Hammes, S. R., and Levin, E. R. (2007) Endocrine Rev. 28, 726–741
13. Zwart, W., de Leeuw, R., Rondaij, M., Neefjes, J., Mancini, M. A., and Michel, R. J. Cell Sci. 123, 1253–1261
14. Ylikomi, T., Bocquel, M. T., Berry, M., Grönemeyer, H., and Chambon, P. (1992) EMBO J. 11, 3681–3694
15. Kumar, R., and Thompson, E. B. (1999) Steroids 64, 310–319
16. Mader, S., Chambon, P., and White, J. H. (1993) Nucleic Acids Res. 21, 1125–1132
17. Tanner, T. M., Denayer, S., Geverts, B., Van Tilborgh, N., Kerkhofs, S., Helsen, C., Spans, L., Dubois, V., Houtsmuller, A. B., Claessens, F., and Haelens, A. (2010) Cell Mol. Life Sci. 67, 1919–1927
18. Xu, P. L., Liu, Y. Q., Shan, S. F., Kong, Y. Y., Zhou, Q., Li, M., Ding, J. P., Xie, Y. H., and Wang, Y. (2004) Mol. Endocrinol. 18, 1887–1905
19. Picard, D., Kumar, V., Chambon, P., and Yamamoto, K. R. (1990) Cell Regul. 1, 291–299
20. Francis, M. K., Pinhey, D. G., and Ryder, K. (1995) J. Biol. Chem. 270, 11502–11513
21. Teyssier, C., Belguise, K., Gallier, F., and Chalbos, D. (2001) J. Biol. Chem. 276, 36361–36369
22. Chambless, K. L., Simon, L., Yuhanna, I. S., Mineo, C., and Shaul, P. W. (2005) Mol. Endocrinol. 19, 277–289
23. Jakacka, M., Ito, M., Martinson, F., Ishikawa, T., Lee, E. J., and Jameson, J. L. (2002) Mol. Endocrinol. 16, 2188–2201
24. Zhang, Z., Maier, B., Santen, R. J., and Song, R. X. (2002) Biochim. Biophys. Acta 1584, 393–399
25. Yue, W., Fan, P., Wang, J., Li, Y., and Santen, R. J. (2007) J. Steroid Biochem. Mol. Biol. 106, 102–110
26. Razandi, M., Alton, G., Pedram, A., Ghonshani, S., Webb, P., and Levin, E. R. (2003) Mol. Cell. Biol. 23, 1633–1646
27. Razandi, M., Pedram, A., Greene, G. L., and Levin, E. R. (1999) Mol. Endocrinol. 13, 307–319
28. Acconcia, F., Ascenzi, P., Bocedi, A., Spinsì, E., Tomasi, V., Tarentalace, A., Visca, P., and Marino, M. (2005) Mol. Biol. Cell 16, 231–237
29. Levin, E. R. (2005) Mol. Endocrinol. 19, 1951–1959
30. Harrington, W. R., Kim, S. H., Funk, C. C., Madak-Erdogan, Z., Schiff, R., Katzenellenbogen, J. A., and Katzenellenbogen, B. S. (2006) Mol. Endocrinol. 20, 491–502
31. Rai, D., Frolova, A., Frasar, J., Carpenter, A. E., and Katzenellenbogen, B. S. (2005) Mol. Endocrinol. 19, 1606–1617
32. Poniglittimongkol, M., Green, S., and Chambon, P. (1988) EMBO J. 7, 3385–3388
33. Winuthayanon, W., Piyaucharurapat, W., Suksamrarn, A., Poniglittimongkol, M., Arao, Y., Hewitt, S. C., and Korach, K. S. (2009) Environmental Health Perspectives 117, 115–116
34. Cousse, J. F., Curtis, S. W., Washburn, T. F., Lindzey, J., Golding, T. S., Lubahn, D. B., Smithies, O., and Korach, K. S. (1995) Mol. Endocrinol. 9, 441–454
35. Mueller, S. O., Hall, J. M., Swope, D. L., Pedersen, L. C., and Korach, K. S. (2003) The J. Biol. Chem. 278, 12255–12262
36. Salmon, P., and Trono, D. (2006) Current Protocols in Neuroscience 37, 4.21.1–4.21.24
37. Pfaffl, M. W. (2001) Nucleic Acids Res. 29, e45
38. la Cour, T., Kiemer, L., Melgaard, A., Gupta, R., Skriver, K., and Brunak, S. (2004) Protein Eng. Des. Sel. 17, 527–536
39. Webb, P., Lopez, G. N., Uht, R. M., and Kushner, P. J. (1995) Mol. Endo-
40. Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997) *Nature* **387**, 733–736.
41. Carrigan, A., Walther, R. F., Salem, H. A., Wu, D., Atlas, E., Lefebvre, Y. A., and Haché, R. I. (2007) *J. Biol. Chem.* **282**, 10963–10971.
42. Cazalla, D., Zhu, J., Manche, L., Huber, E., Krainer, A. R., and Cáceres, J. F. (2002) *Mol. Cell. Biol.* **22**, 6871–6882.
43. Nakielny, S., and Dreyfuss, G. (1996) *J. Cell Biol.* **134**, 1365–1373.
44. Krum, S. A., Miranda-Carboni, G. A., Lupien, M., Eckhoute, J., Carroll, J. S., and Brown, M. (2008) *Mol. Endocrinol.* **22**, 2393–2406.
45. Huq, M. D., Ha, S. G., and Wei, L. N. (2008) *J. Proteome Res.* **7**, 4538–4545.
46. Subramanian, K., Jia, D., Kapoor-Vazirani, P., Powell, D. R., Collins, R. E., Sharma, D., Peng, J., Cheng, X., and Vertino, P. M. (2008) *Mol. Cell* **30**, 336–347.
47. Fu, M., Wang, C., Reutens, A. T., Wang, J., Angeletti, R. H., Siconolfi-Baez, L., Ogryzko, V., Avantaggiati, M. L., and Pestell, R. G. (2000) *J. Biol. Chem.* **275**, 20853–20860.
48. Sentis, S., Le Romancer, M., Bianchin, C., Rostan, M. C., and Corbo, L. (2005) *Mol. Endocrinol.* **19**, 2671–2684.
49. Shaffer, P. L., McDonnell, D. P., and Gewirth, D. T. (2005) *Biochemistry* **44**, 2678–2685.
50. Yoshikawa, N., Shimizu, N., Sano, M., Ohnuma, K., Iwata, S., Hosono, O., Fukuda, K., Morimoto, C., and Tanaka, H. (2008) *Biochem. Biophys. Res. Commun.* **371**, 44–49.
51. Hong, W., Banaihmad, A., Liu, Y., and Li, H. (2008) *J. Mol. Biol.* **384**, 22–30.
52. Zhou, Q., Shaw, P. G., and Davidson, N. E. (2009) *Endocrine-Rel. Cancer* **16**, 319–323.
53. Wang, C., Fu, M., Angeletti, R. H., Siconolfi-Baez, L., Reutens, A. T., Albanese, C., Lisanti, M. P., Katzenellenbogen, B. S., Kato, S., Hopp, T., Fuqua, S. A., Lopez, G. N., Kushner, P. J., and Pestell, R. G. (2001) *J. Biol. Chem.* **276**, 18375–18383.
54. Kim, M. Y., Woo, E. M., Chong, Y. T., Homenko, D. R., and Kraus, W. L. (2006) *Mol. Endocrinol.* **20**, 1479–1493.
55. Pedram, A., Razandi, M., Sainson, R. C., Kim, J. K., Hughes, C. C., and Levin, E. R. (2007) *J. Biol. Chem.* **282**, 22278–22288.
56. Zhang, Z., Cergnet, M., Mullins, C., Williamson, M., Bessert, D., and Skoff, R. (2004) *J. Neurochem.* **89**, 674–684.