Acetylation of Estrogen Receptor α by p300 at Lysines 266 and 268 Enhances the Deoxyribonucleic Acid Binding and Transactivation Activities of the Receptor

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Using a variety of biochemical and cell-based approaches, we show that estrogen receptor α (ERα) is acetylated by the p300 acetylase in a ligand- and steroid receptor coactivator-dependent manner. Using mutagenesis and mass spectrometry, we identified two conserved lysine residues in ERα (Lys266 and Lys268) that are the primary targets of p300-mediated acetylation. These residues are acetylated in cells, as determined by immunoprecipitation-Western blotting experiments using an antibody that specifically recognizes ERα acetylated at Lys266 and Lys268. The acetylation of ERα by p300 is reversed by native cellular deacetylases, including trichostatin A-sensitive enzymes (i.e. class II deacetylases, such as sirtuin 1). Acetylation at Lys266 and Lys268, or substitution of the same residues with glutamine (i.e. K266/268Q), a residue that mimics acetylated lysine, enhances the DNA binding activity of ERα in EMSAs. Likewise, substitution of Lys266 and Lys268 with glutamine enhances the ligand-dependent activity of ERα in a cell-based reporter gene assay. Collectively, our results implicate acetylation as a modulator of the ligand-dependent gene regulatory activity of ERα. Such regulation is likely to play a role in estrogen-dependent signaling outcomes in a variety of estrogen target tissues in both normal and pathological states. (Molecular Endocrinology 20: 1479–1493, 2006)

THE GENE-REGULATORY ACTIONS of estrogens are mediated through two nuclear estrogen receptor (ER) proteins, ERα and ERβ, which belong to the nuclear receptor superfamily (1–3). ERs bind estrogens with high affinity and function as ligand-regulated transcription factors to control global patterns of gene expression. ERs and ERβ have unique, but overlapping, patterns of expression in a variety of estrogen target tissues, including mammary glands, uterus, and bone (4–6). In addition, the two ERs may exhibit distinct gene-regulatory activities under certain promoter and cell contexts (7–15).

ERα and ERβ share a conserved structural and functional organization, including the following: 1) an amino-terminal A/B region containing a transcriptional activation function (AF-1), 2) a DNA-binding domain (DBD), and 3) a carboxyl-terminal ligand-binding domain (LBD) containing a second transcriptional activation function (AF-2) (see Fig. 2B) (1, 3). In addition to these canonical nuclear receptor domains, recent studies have also begun to characterize the C-terminal extension (CTE) of the ERα and ERβ DBDs (amino acids 251–288 and 170–207, respectively), which plays a role in regulating the DNA-binding activities of the receptors (16, 17). The coordinated actions of the aforementioned ER functional domains allow for precisely controlled signal-regulated transcription in response to both natural and synthetic ER ligands.

The binding of agonist ligands by ERα and ERβ promotes a conformational change in the receptor LBD that allows the receptor to interact directly or indirectly with a diverse set of coregulatory proteins (1, 3). These include members of the steroid receptor coactivator (SRC) family (i.e. SRC1,-2, and -3), which function primarily as bridging factors to recruit other coregulators (18–20), including a diverse set of pro-
tein-modifying enzymes (e.g. acetylases, methyltransferases, kinases) (21, 22). p300 and its paralog cAMP response element binding protein-binding protein (CBP) are the two best characterized mammalian acetylases. They function as coregulators for a variety of transcription factors, including ERs and other nuclear receptors (23–25). p300 and CBP are recruited to ERα and ERβ in a ligand-dependent manner via interactions with SRC proteins. During an estrogen-dependent transcriptional response, p300 and CBP can acetylate nucleosomal histones to alter chromatin structure and function (19, 22), as well as components of the transcription complex to alter transcriptional activity (see below). A variety of deacetylases, including trichostatin A (TSA)-sensitive enzymes (i.e. class I and II deacetylases, such as histone deacetylase 1) and nicotinamide adenine dinucleotide (NAD⁺)-dependent/nicotinamide-sensitive enzymes (i.e. class III deacetylases, such as sirtuin 1 (SIRT1)), also function as coregulators and can reverse the protein acetylation reactions catalyzed by acetylases (26–29).

A number of recent studies have shown that acetylation is an important covalent posttranslational modification for regulating the activity of transcription-related factors, including p53, SRC3 (also known as ACTR), nuclear factor-κB p65, and poly(ADP-ribose) polymerase-1 (30–39). Interestingly, different acetylases (e.g. p300/CBP vs. p300/CBP-associated factor) have different substrate preferences as demonstrated by the fact that some factors can be acetylated by one but not the other (35, 36, 38, 40). With regard to modulating the activity of transcription-related factors, the consequence of acetylation may be either transcriptional activation or inhibition. Acetylation of transcription-related factors can increase their transcriptional activity by the following: 1) enhancing DNA binding activity, 2) stimulating interactions with positive transcriptional regulators, such as chromatin remodeling factors or coactivators, 3) inhibiting interactions with negative regulators, resulting in a loss of transcription repression, 4) increasing the stability of the factors, and 5) altering subcellular localization (35, 36, 38, 40). Likewise, acetylation may inhibit the activity of transcription-related factors by reducing binding to DNA or chromatin, as well as reducing protein–protein interactions required for transcriptional activation (35, 36, 38, 40). Thus, the specific biochemical effects of acetylation are varied and differ with each target protein.

In the studies described herein, we show that ERα, but not ERβ, is a target for acetylation by p300. Using a variety of biochemical and cell-based assays, we have identified the sites of acetylation, and explored the mechanisms and functional consequences of p300-mediated acetylation on ERα activity. Collectively, our results implicate acetylation as modulator of the ligand-dependent gene regulatory activity of ERα.

RESULTS

Human ERα Is Acetylated by p300 and Deacetylated by TSA- and Nicotinamide-Sensitive Deacetylases

To examine the acetylation of human ERα by p300, we used an in vitro acetylation assay with [³H]acetate co-enzyme A (acetyl CoA) and the following purified recombinant proteins: ERα, p300, and glutathione S-transferase (GST)-fused SRC2(RID/PID), which contains the receptor interaction domain (RID) and p300/CBP interaction domain (PID) of SRC2 (Fig. 1A). With this assay, we showed previously that SRCs play a key role in the targeted acetylation of nucleosomal core histones by p300, functioning as a bridging factor between p300 and 17β-estradiol (E2)-bound ERα (Ref. 41 and Fig. 1B, bottom). Interestingly, these same interactions also promote the acetylation of ERα (Fig. 1B, top), indicating that interactions among agonist-bound ERα, SRC, and p300 are required for efficient acetylation of ERα by p300 (see also supplemental Fig. 1, published as supplemental data on The Endocrine Society’s Journals Online web site at http://mend.endojournals.org). SRC2(RID/PID), which lacks the putative SRC acetylase domain (18, 21), was unable to promote the acetylation of ERα in the absence of p300 (data not shown).

Next, we examined whether the p300-dependent acetylation of ERα can be reversed by native deacetylases present in a HeLa cell nuclear extract (HNE). Immobilized [³H]acetylated ERα was incubated with HNE, which contains a variety of deacetylases including TSA-sensitive enzymes (i.e. class I and II deacetylases) and NAD⁺-dependent/nicotinamide-sensitive enzymes (i.e. class III deacetylases, such as SIRT1) (26–29). As shown in Fig. 1C, incubation with HNE dramatically reduced the acetylation of ERα (compare lanes 1 and 2). The addition of TSA inhibited deacetylation of ERα by the HNE (lane 3), indicating that one or more class I/II deacetylases present in the HNE can deacetylate ERα. The addition of NAD⁺ in the presence of TSA (i.e. under conditions in which class I/II deacetylases were inhibited) also resulted in the deacetylation of ERα (compare lanes 3 and lane 4). The effect of NAD⁺ was blocked by the addition of nicotinamide (lane 5), indicating that one or more class III deacetylases present in the HNE, such as SIRT1, can deacetylate ERα. To explore this last result further, we performed a similar set of experiments using purified recombinant SIRT1 (Fig. 1A) in place of the HNE. As shown in Fig. 1D, SIRT1 without added NAD⁺ had no effect on the acetylation of ERα (compare lanes 1 and 2), whereas SIRT1 in the presence of NAD⁺ was a potent deacetylase of ERα (lane 3). The deacetylation of ERα by SIRT1 plus NAD⁺ was inhibited by nicotinamide (lane 4), but not by TSA (lane 5). Taken together, these results indicate that the acetylation of ERα by p300 can be reversed by native TSA- and nicotinamide-sensitive deacetylases, including SIRT1.
Initial Identification of Lys268 and Lys266 as Sites of Acetylation in ERα

Full-length ERα contains 29 lysine residues that are potential sites of acetylation by p300: four in the A/B region, 10 in the DBD, and 15 in the LBD (for the purposes of this study, the domains have the boundaries defined in Fig. 2A). To identify the lysine residues in ERα that are acetylated by p300, we used deletion mutants (Fig. 2B and C), point mutants (Fig. 3), and an unbiased quantitative mass spectrometry approach (Fig. 4). ERα deletion mutants lacking either the A/B region (i.e., ERαΔAB), DBD (i.e., ERαΔDBD), or both the A/B region and the DBD (i.e., GST-LBD[282–595]) (Fig. 2B) were assayed for E2- and SRC-dependent acetylation by p300 in the presence of [3H]acetyl CoA. Importantly, all three of these receptor deletions contain intact LBDs and SRC interaction domains, because efficient acetylation of ERα requires the binding of E2 and SRC (Fig. 1B and supplemental Fig. 1). Note that, for these assays, we used equal molar amounts of the purified receptor proteins, as opposed to equal mass amounts (Fig. 2C, bottom), so that the relative number of [3H]acetyl groups added per mole of protein could be assessed. The ERα deletion mutant lacking the A/B region (i.e., ERαΔAB) showed a modest reduction in E2- and SRC-dependent acetylation by p300, whereas the ERα deletion mutants lacking the DNA binding domain (i.e., ERαΔDBD and GST-LBD[282–595]) showed a dramatic reduction in acetylation (Fig. 2C, top). These results suggested that the DBD is the major target for acetylation by p300. This result was confirmed by the independent approaches described below.

To determine the sites of acetylation more precisely, we individually changed the 10 lysine residues in the DBD and the four lysine residues in the A/B region to arginine, an amino acid that has a positively charged side chain like lysine, but cannot be acetylated. The 14 lysine to arginine (KfR) point mutant ERαs were expressed and purified as recombinant proteins, and then assayed for E2- and SRC-dependent acetylation vs. wild-type ERα. Of the 14 KfR point mutants tested, only two showed a reduction in acetylation: K268R (~50% reduction) and K266R (~20% reduction) (Fig. 3A and data not shown). When both Lys266 and Lys268 were mutated, there was a dramatic (~90%) reduction in acetylation (Fig. 3B). These results provided a first indication that Lys268 and Lys266, both of which are in the CTE of the DBD (17), are major and minor targets, respectively, for E2- and SRC-dependent acetylation by p300.

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Fig. 1. ERα Is Acetylated by p300 and Deacetylated by TSA- and Nicotinamide-Sensitive Deacetylases

A, SDS-PAGE analyses of purified recombinant ERα, GST-SRC2(RID/PID), p300, and SIRT1 stained with Coomassie brilliant blue R-250. Size markers in kilodaltons are shown. The asterisk for the GST-SRC2(RID/PID) sample indicates a major breakdown product with minor breakdown products below. B, p300 acetylates ERα and nucleosomal core histones in an E2- and SRC-dependent manner. ERα and salt-dialyzed chromatin were incubated with p300 in the presence of GST-SRC2(RID/PID), E2, and [3H]acetyl CoA. The reactions were analyzed by SDS-PAGE with subsequent fluorography. C, ERα is deacetylated by TSA- and nicotinamide-sensitive deacetylases. [3H]AcERα was incubated with HeLa cell nuclear extract (HeLa NE) in the presence or absence of NAD+, TSA, or nicotinamide as indicated. The reactions were analyzed by SDS-PAGE with subsequent fluorography. D, ERα is deacetylated by the NAD+–dependent deacetylase SIRT1. The assays were set up as in C except that purified recombinant SIRT1 was used in place of the HeLa cell NE.
Confirmation by Mass Spectrometry of Lys268 and Lys266 as Sites of Acetylation in ERα

To confirm the sites of acetylation in ERα using an independent and unbiased assay, we used the quantitative mass spectrometry approach outlined in Fig. 4A (42). In vitro acetylation reactions with full-length ERα plus SRC, p300, and E2 were performed in the absence or presence of cold acetyl CoA. After enzymatic acetylation of the lysine residues in ERα specifically targeted by p300, all of the remaining unacetylated lysine residues were chemically acetylated using deuterated-acetic anhydride. The modified ERα protein was then digested with trypsin. The resulting peptides contained light acetyl groups (AcK) on lysines acetylated by p300 and heavy (deuterated) acetyl groups (DAcK) on all other lysines. The mass difference of 3 Da between AcK and DAcK on the peptides was visualized by matrix-assisted laser desorption/ionization (MALDI)-quadrupole-quadrupole-time of flight (QqTOF) mass spectrometry (43). The percent acetylation of the chemically identical, isotopically distinct peptides (i.e. heavy vs. light) was calculated directly from ratios of the intensities of the monoisotopic peaks (42).

The mass spectrometry approach provided good coverage of the lysine residues in the A/B region, DBD, and hinge region spanning amino acids 1 through 298 (12 of the 14 lysine residues in this region were detected and quantified; Lys48 and Lys257 were in pep-
tides that were not observed by the mass spectrometry approach) (supplemental Table 1). Coverage in the LBD spanning amino acids 299 through 595 was less complete (only eight of the 15 lysine residues in this region were detected and quantified) (supplemental Table 1). However, the results in Fig. 2C indicate that the LBD is not a major target of acetylation by p300. Furthermore, when taken in combination, the three analytical approaches that we used (i.e. deletion mutants, point mutants, and mass spectrometry) yielded complete coverage of all 29 lysine residues in ERα (supplemental Table 1).

Fig. 3. Mutation of Lys266 and Lys268 in ERα Reduces Acetylation by p300

A, Acetylation assays with ERα lysine mutants. Purified FLAG-tagged wild-type and Lys to Arg single-point mutant ERαs were assayed for acetylation by p300 in the presence of GST-SRC2(RID/PID) and E2 as described for Fig. 1B. Top, SDS-PAGE analysis of the purified ERα proteins with subsequent staining using Coomassie brilliant blue R-250 to confirm equal protein amounts. Size markers in kilodaltons are shown. Bottom, Summary of the results from the acetylation assays. The ERα bands were excised from the gels after fluorography and quantified by liquid scintillation counting. The acetylation level of each lysine point mutant was expressed relative to wild type. Each bar represents the mean ± SEM from at least three different experiments. B, Acetylation assays with Lys to Arg 266/268 double-point mutant ERαs. Wild-type and mutant ERαs were assayed for acetylation by p300 in the presence of GST-SRC2(RID/PID) and E2 as described for Fig. 1B. Top, SDS-PAGE analysis of the purified ERα proteins with subsequent fluorography. Bottom, The same gel stained using Coomassie brilliant blue R-250 to confirm equal protein amounts.

Three of the detectable tryptic peptides from the mass spectrometry analysis had quantifiable levels of acetyl CoA-dependent acetylation (expressed as a percentage of the total amount of each peptide present in the reaction) (Table 1). The acetylated peptides spanned the following residues in ERα: 1) 244–256, containing Lys244 and Lys252, 2) 264–269, containing Lys266 and Lys268, and 3) 288–300, containing Lys299 (Table 1). The 264–269 peptide had approximately 3- and 6-fold more total p300-dependent acetylation than the 244–256 and 288–300 peptides, respectively (Table 1; 31.5 ± 0.9 vs. 11.1 ± 0.7 and 4.5 ± 0.6%, respectively), indicating that the 264–269 peptide contains the major target for E2- and SRC-dependent acetylation by p300. These results fit well with our mutagenesis experiments, which identified Lys268 and Lys266 as major and minor sites of acetylation, respectively. Based on these results, we focused on Lys266 and Lys268 and explored the E2- and SRC-dependent acetylation of these residues by p300 in more detail.

Mass spectrometry/mass spectrometry (MS/MS) analysis using MALDI-ion trap mass spectrometry was used to quantify the acetylation of the two individual lysine residues in the 264–269 peptide (i.e. Lys266 and Lys268). For the singly acetylated species of the 264–269 peptide, >95% of the acetylation was on Lys268 (Table 1 and data not shown). In addition, the singly acetylated (i.e. primarily Ac-Lys268) species of the 264–269 peptide was present at approximately 3- to
4-fold more than the doubly acetylated (i.e., Ac-Lys266 plus Ac-Lys268) species (Fig. 4B and Table 1). Furthermore, mutation of Lys268 to an unacetylatable residue (i.e., glutamine, Q) reduced the amount of both single and double acetylation of the 264–269 peptide (Fig. 4B), even though a dramatic (~10-fold) compensatory increase in the acetylation of Lys266 was observed, from ~1% of the total peptide for wild-type ERα (Table 1, see Single Acetylation column for K266) to ~10% of the total peptide for the K268Q mutant (Fig. 4B). Further MS/MS analyses demonstrated that both single and double acetylation of the 264–269 peptide occurred efficiently only when acetylation of the full-length ERα was carried out in the presence of E2 and GST-SRC2(RID/PID) (Fig. 4B). Collectively, the mass spectrometry data support the conclusions that 1) Lys268 is the major site of E2- and SRC-dependent acetylation by p300 and 2) Lys266 is a minor site whose acetylation can be increased by mutation or prior acetylation of Lys268. These conclusions fit well with the conclusions from the mutagenesis studies described above.

Lys302 and Lys303 Are Not Major Sites of Acetylation by p300 in Full-Length ERα

Results from a recent study by Wang et al. (44) showed that Lys302 and Lys303 in an isolated fragment of ERα (i.e., amino acids 282–420) can be acetylated by p300 in the absence of E2 and SRC. We examined whether these same sites might be potential sites of acetylation in full-length ERα by mutating both sites to arginine, a residue that cannot be acetylated. We found no difference in the acetylation of wild-type or K302/303R ERα by p300 with or without E2 or GST-SRC2(RID/PID) (Figs. 2C and 3A; data not shown), indicating that Lys302 and Lys303 are not targets for acetylation by p300 in full length of ERα. These results were confirmed in our mass spectrometry analysis, which showed no detectable acetylation on Lys302 and Lys303 (Table 1, supplemental Table 1, and data not shown). Interestingly, a fragment of ERα containing the entire LBD including Lys302 and Lys303 (i.e., amino acids 282–595) was not acetylated by p300 (Fig. 2C, lanes 9 and 10), yet the smaller fragment used by Wang et al. (44) (i.e., amino acids 282–420) was acetylated (Fig. 2C, lanes 11 and 12). These results suggest that Lys302 and Lys303 are cryptic residues that are not normally accessible to p300 in full-length ERα. Truncation of the LBD (as in the 282–420 fragment) presumably disrupts the secondary and tertiary structure of the LBD, which could cause Lys302 and Lys303 to become exposed and accessible to acetylation by p300. Taken together, our results indicate that Lys302 and Lys303 are not major sites of acetylation by p300 in full-length ERα.

Lys268 and Lys266 Are Conserved across Species

Alignment of human ERα with the ERαs from a variety of other species shows that Lys268 and Lys266 have been conserved throughout evolution, at least from amphibians through mammals (Fig. 5A). Furthermore, three of the four fish species examined have at least one lysine in the same position as Lys266 and Lys268, or within one residue (Fig. 5A). Although alignment of human ERα with other human nuclear receptors is difficult for the CTE due to low sequence conservation, the alignment can be fixed at the most carboxyl-terminal conserved cysteine residue in the second zinc

Fig. 4. Quantitative Mass Spectrometric Analysis of ERα Acetylation by p300

A, Schematic diagram of the quantitative mass spectrometric procedure for determining site-specific ERα acetylation levels. B, Quantitative mass spectrometric determination of SRC- and E2-dependent acetylation by p300 of peptide 264–269 from trypsin-digested full-length wild-type or K268Q mutant ERα. The ERαs were assayed for acetylation by p300 in the presence of GST-SRC2(RID/PID) and E2 as described for Fig. 1B and then subjected to quantitative MALDI-QqTOF spectrometry as described in A. The data are expressed as amount of acetylated 264–269 peptide (i.e., AcK) relative to the total amount of 264–269 peptide in the reaction (i.e., AcK plus DAcK). As indicated in Table 1, K268 is the major site (~95%) of single acetylation in the 264–269 peptide. In the K268Q mutant, all of the acetylation of the 264–269 peptide is at K266.
finger of the DBD (Cys240 for ERα; Fig. 5B). Such an analysis reveals that almost all non-orphan nuclear receptors have at least one, but typically two or more, and as many as six, lysine residues located in the amino-terminal portion of the CTE (+11 to +32 relative to the aforementioned conserved cysteine residue). This region includes the three lysine residues in the androgen receptor (AR) that are acetylated by p300/CBP (Lys630, Lys632, and Lys633) (45–47). These lysine-rich regions may be targeted for acetylation in other receptors as well. Interestingly, ERβ lacks lysine residues homologous to Lys266 and Lys268 in ERα (Fig. 5B), fitting well with our observation that ERβ is not acetylated by p300 under the same conditions that lead to acetylation of ERα (data not shown).

**Lys268 and Lys266 of ERα Are Acetylated in Vivo**

Next, we determined whether Lys266 and Lys268 are bona fide acetylation sites in vivo. For these studies, we generated an antiserum that specifically recognizes acetylated Lys266/Lys268 ERα (Ack266/268-ERα) using a doubly acetylated peptide antigen spanning amino acids 263 through 273 of human ERα. Due to sequence conservation among mammalian ERαs (Fig. 5A), this antiserum should recognize similarly acetylated ERα from most, if not all, mammalian species. As shown in the Western blots in Fig. 6A, this antiserum specifically recognizes purified wild-type ERα that has been acetylated by p300 (middle panels), but not unacetylated wild-type ERα (left panels). Furthermore, this antiserum does not recognize K266/268Q ERα (right panels) or K268Q ERα (data not shown) that has been incubated with p300 under conditions that lead to the acetylation of wild-type ERα (i.e., acetyl CoA, +E2, +SRC). These results, as well as the assays described below, demonstrate that this antiserum specifically recognizes acetylated K266/268 ERα.

Once we verified the specificity of our antiserum, we carried out in vivo acetylation assays by using the experimental scheme illustrated in Fig. 6B. Briefly, FLAG-tagged ERα was immunoprecipitated from one of two cell lines (231/ERα cells, which stably express FLAG-tagged ERα, or transfected 293T cells, which transiently express FLAG-tagged ERα). Note that the cells were treated with TSA and nicotinamide before collection to block the actions of deacetylases during the experiment. In the absence of these treatments, the acetylation of ERα was dramatically reduced (data not shown), suggesting that deacetylation of the receptor occurs rapidly in vivo. The immunoprecipitates were then analyzed for acetylated ERα using the acetylated Lys266/Lys268 ERα antiserum described above. To demonstrate acetylation dependence for the observed signal, the immunoprecipitated material was deacetylated by the addition of recombinant SIRT1 plus NAD⁺ in some cases before Western blotting.

In 231/ERα cells, we observed basal acetylation of Lys266/Lys268 that was increased about 2- to 3-fold in the presence of E2 (Fig. 6C, compare lanes 1 and 2). As expected, the signal for acetylated Lys266/Lys268 ERα in the immunoprecipitated material was lost upon incubation with SIRT1 plus NAD⁺ before Western blotting (compare lanes 3 and 4). These results indicate that ERα from a well-characterized estrogen-responsive cell line that stably expresses the receptor (for example, see Refs. 48 and 49) is acetylated at Lys266 and Lys268, and that the level of acetylation can be modulated by E2. Next, we used transient expression of FLAG-tagged ERα in 293T cells, which are ERα negative, so that we could compare the acetylation of wild-type ERα with an unacetylatable Lys266/Lys268 mutant (i.e., K266/268R) in vivo. Wild-type ERα immunoprecipitated from the transfected 293T cells was acetylated at Lys266/Lys268 (Fig. 6D, lane 1). Again, as expected, the signal for acetylated Lys266/Lys268 ERα was lost upon incubation of the immunoprecipitated material with SIRT1 plus NAD⁺ before Western blotting (lane 2). In contrast to wild-type ERα, no acet-

**Table 1. Quantitative Mass Spectrometric Analysis of ERα Reveals Lys268 as a Major Site of Acetylation by p300**

<table>
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<tr>
<th>ERα Peptide (Lysines)</th>
<th>Total Acetylation (%)</th>
<th>Single Acetylation (%)</th>
<th>Double Acetylation (%)</th>
<th>Major Site of Acetylation (%)</th>
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<tr>
<td>244–256 (K244, K252)</td>
<td>11.1 ± 0.7</td>
<td>10.4 ± 0.9 (–10.1% from K252, –0.3% from K244)</td>
<td>0.7 ± 0.4</td>
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<tr>
<td>264–269 (K266, K268)</td>
<td>31.5 ± 0.9</td>
<td>24.7 ± 0.9 (–23.5% from K268, –1.2% from K266)</td>
<td>6.8 ± 0.9</td>
<td>K268 (~95%)</td>
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<tr>
<td>288–300 (K299)</td>
<td>4.5 ± 0.6</td>
<td>4.5 ± 0.6</td>
<td>N/A</td>
<td>K299 (100%)</td>
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* a Full-length ERα was acetylated by p300 in the presence of SRC2(RID/PID) and E2. The acetylated ERα was then digested with trypsin and subjected to quantitative mass spectrometry as described in Materials and Methods. The percentage of the indicated peptide that was acetylated in an acetyl CoA-dependent manner was determined by comparing the results from acetylation reactions run with and without added acetyl CoA. Note that only the three peptides listed showed appreciable acetyl CoA-dependent acetylation by p300 in the mass spectrometry analysis. The values represent the mean ± SEM for three separate determinations.

b As determined by MS/MS analysis using MALDI-ion trap mass spectrometry. The numbers in parentheses indicate the percentage of the singly acetylated peptide that was acetylated at the site listed.
ylation of the K266/268R mutant was observed (Fig. 6D, lanes 3 and 4). Together, these cell-based studies using an antiserum that specifically recognizes acetylated Lys266/Lys268 ERα/H9251 clearly demonstrate that ERα/H9251 is acetylated at Lys266 and Lys268 in vivo.

Interestingly, similar experiments using an antiserum that specifically recognizes ERα singly acetylated at Lys266 (AcK266-ERα) or Lys268 failed to detect acetylated ERα in the immunoprecipitated material from 231/ERα/H9251 cells and transfected 293T cells (data not shown). These results suggest that, although the AcK268-ERα species may predominate in the in vitro reaction (Table 1), the AcK266/268-ERα species predominates in cells.

Acetylation or Mutation of ERα at Lys266/Lys268 Does Not Affect E2 Binding, Interaction with SRC2, or Subcellular Localization of ERα

Our initial studies using 1) a transcriptionally inactive ERα mutant defective in SRC binding (i.e. L540Q) and 2) a set of previously characterized polypeptide inhibitors that block ERα-SRC and SRC-p300/CBP interactions showed that acetylation of ERα by p300 correlates with E2-dependent transcriptional activation (supplemental Fig. 1). To explore the possible effects of acetylation on the activities of ERα in more detail, we performed a number of functional assays to examine ligand binding (supplemental Fig. 2A), ERα-SRC2

Fig. 5. Alignment of the Amino Acid Sequences Surrounding Lys266 and Lys268 in Human ERα with Corresponding Regions from Other ERαs and Other Nuclear Receptors

Sequence alignment of the CTE of the human ERα DBD with the corresponding region of ERs from other species and other nuclear receptors. The alignments were anchored at the last conserved cysteine residue in the second zinc finger of the DBD (first C in each row). The boxed region demarcates the amino-terminal portion of the CTE [i.e. those residues located within +11 to +32 of the aforementioned cysteine residue; note that the full CTE, as defined by Melvin et al. (17), extends to +47]. Asterisks indicate residues that are conserved in all of the receptors shown. A, Alignment of ERαs. The lysine residues (K) highlighted in bold correspond to Lys266 and Lys268 in human ERα. B, Alignment of human nuclear receptors. All of the lysine residues (K) in the boxed region are highlighted in bold. K266 and K268 of ERα, and K630, K632, and K633 of AR are underlined.

<table>
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<th>Species</th>
<th>Sequence Alignment</th>
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<tr>
<td>Pig</td>
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<tr>
<td>Rainbow Trout</td>
<td>CRLRKYDVKGKQIRKDRGGRMLLHHRQRD</td>
</tr>
<tr>
<td>Catfish</td>
<td>CRLRKYDVKGKQIRKDRGGRMLLHHRQRD</td>
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<tr>
<td>Tilapia</td>
<td>CRLRKYDVKGKQIRKDRGGRMLLHHRQRD</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>CRLRKYDVKGKQIRKDRGGRMLLHHRQRD</td>
</tr>
</tbody>
</table>

Asterisks indicate residues that are conserved in all of the receptors shown.
interactions (supplemental Fig. 2B), subcellular localization (supplemental Fig. 2C), DNA binding (Fig. 7), and transactivation (Fig. 8). For these assays, we used a set of Lys266/Lys268 double mutants in which the lysine residues were changed to arginine (R, which has a positively charged side chain and mimics unacetylated lysine) or glutamine (Q, which has a neutral side chain and mimics acetylated lysine). As shown in Fig. 3B, ERα/H9251 mutants harboring these changes (i.e. K266/268R and K266/268Q) showed a dramatic reduction in acetylation by p300 in vitro compared with wild-type ERα/H9251 (see also Fig. 6, A and D). Our results indicate that acetylation or mutation of ERα/H9251 at Lys266/Lys268 does not appreciably affect E2 binding, interaction with SRC2, or subcellular localization of ERα (supplemental Fig. 2).

Acetylated ERα and K266/268Q ERα Exhibit Increased DNA Binding Activity

Next, we examined the effects of acetylation on ERα DNA binding activity by using EMSAs. In our initial assays, purified wild-type ERα was incubated with p300 in the presence of E2 and GST-SRC2(RID/PID) with or without unlabeled acetyl CoA to generate acetylated or unacetylated ERα, respectively. Aliquots of each reaction were subjected to Western blotting using the following: 1) an anti-acetyl-lysine antibody to confirm the acetylation of ERα, which occurred only in the presence of acetyl CoA, as expected (Fig. 7A, top) and 2) an anti-ERα antibody, which detected total ERα (Fig. 7A, middle). Another aliquot from each reaction was used in EMSAs with a 32P-labeled double-stranded DNA probe containing an ERE sequence (Fig. 7A, bottom). Interestingly, acetylated ERα showed an approximate 4-fold increase in DNA binding activity compared with mock acetylated ERα (i.e. reaction without acetyl CoA). This effect was blocked by mutation of Lys266/Lys268 to arginine, but not Lys180, which was used as a control (Fig. 7B). Interestingly, mutation of Lys266/Lys268 to glutamine (i.e. K266/268Q), but not arginine (i.e. K266/268R), increased DNA binding activity of unacetylated ERα by about 5-fold (Fig. 7C). Thus, ERα DNA binding activity increases as the side chain charge at residues 266 and 268 are neutralized (from +1 at lysine to neutral at glutamine). Together, these results indicate that acetylation at Lys266/Lys268 can play a role in regulating the DNA binding activity of ERα.
creased DNA Binding Activity

the DNA binding activity of ER

Acetylated Lys) inhibits acetylation-dependent increases in

antibody (were subjected to Western blotting using an acetyl lysine

unlabeled acetyl CoA as indicated. Aliquots of the reactions

incubated with p300, GST-SRC2(RID/PID), and E2 in the

binding activity in EMSAs. Aliquots of purified ER

K266/268Q and K266/268R mutant ER

Hanced transactivation, we tested the activity of the

ing activity observed in the EMSAs might lead to en-

Finally, to determine whether the increased DNA bind-

K266/268Q ER

in Cells

Fig. 7. Acetylated ERα and K266/268Q ERα Exhibit Increased DNA Binding Activity

A, Acetylation of ERα by p300 in vitro increases ERα DNA binding activity in EMSAs. Aliquots of purified ERα were incubated with p300, GST-SRC2(RID/PID), and E2 in the absence (i.e. no acetylation) or presence (i.e. acetylation) of unlabeled acetyl CoA as indicated. Aliquots of the reactions were subjected to Western blotting using an acetyl lysine antibody (top) or an ERα antibody (middle), or analyzed by EMSA (bottom). The fold increase of ERα:ERE complex formation upon acetylation is indicated. B, Changing the p300 acetylation sites in ERα from Lys to Arg (which mimics unacetylated Lys) inhibits acetylation-dependent increases in the DNA binding activity of ERα in EMSAs. The assays were set up as described for A using wild-type, K180R, and K266/268R mutant ERα, followed by an EMSA. The fold increase of ERα:ERE complex formation upon acetylation is indicated. C, Changing Lys266 and Lys268 in ERα to Glu (Q) increases the DNA binding activity of ERα in EMSAs. The EMSAs were set up using increasing amounts of unacetylated wild-type, K180R, and K266/K268Q ERα. The DNA binding activities of the mutant ERα were calculated relative to wild-type ERα. Each bar represents the mean ± SEM from at least three separate determinations.

K266/268Q ERα, But Not K266/268R ERα, Exhibits Increased Transactivation Activity in Cells

Finally, to determine whether the increased DNA binding activity observed in the EMSAs might lead to enhanced transactivation, we tested the activity of the K266/268Q and K266/268R mutant ERα using a cell-based reporter gene assay. Briefly, HeLa cells grown in estrogen-free medium were transfected with a vector for the expression of wild-type, K266/268Q, or K266/268R ERα and a luciferase reporter construct containing two EREs upstream of the estrogen-regulated P2 promoter. After treatment with vehicle or E2 for 18 h, the cells were collected, extracts were prepared, luciferase activity was measured (Fig. 8A), and relative ERα levels were determined by Western blotting (Fig. 8B; no differences were observed). The K266/268Q mutant, which mimics acetylated ERα, gave a 2-fold increase in reporter gene activity relative to wild-type ERα (Fig. 8A), suggesting that acetylation at Lys266/Lys268 can play a role in regulating the transcriptional activity of ERα. In contrast, the K266/268R mutant, which mimics unacetylated ERα, gave reporter gene activity similar to wild-type ERα (Fig. 8A). The lack of an inhibitory effect with the K266/268R mutant may be a consequence of the limited sensitivity of our reporter gene assays, as well as the extent of acetylation required for an observable effect. With regard to this latter point, note that with wild-type ERα, perhaps as few as 5% of the receptor molecules in the cells are acetylated (data not shown), whereas with the K266/268Q mutant, 100% of the receptor molecules are “acetylated.” Collectively, our results demonstrate...
a good correlation between the increased DNA binding and transactivation activities of the K266/268Q mutant ERα, when compared with wild-type ERα.

**DISCUSSION**

**Acetylation Modulates the Activity of ERα, Androgen Receptor, and Possibly Other Nuclear Receptors**

In the studies described herein, we have shown that ERα is acetylated by p300 at Lys266 and Lys268 in an SRC-dependent manner (Figs. 1B, 3, and 4), with Lys268 being the major site of acetylation in full-length ERα (Fig. 4 and Table 1). The extent of ERα acetylation is regulated by E2 both *in vitro* and *in vivo* (Figs. 1B and 6C). In addition, the acetylation of ERα is reversed by native cellular deacetylases, including TSA-sensitive enzymes (*i.e.* class I and II deacetylases) and NAD+/nicotinamide-sensitive enzymes (*i.e.* class III deacetylases, such as SIRT1) (Fig. 1, C and D). Furthermore, our results indicate that acetylation at Lys266 and Lys268 regulates the DNA binding and transcriptional activities of ERα (Figs. 7 and 8). Collectively, our results implicate acetylation as modulator of the ligand-dependent gene regulatory activity of ERα. Ultimately, such regulation is likely to play a role in E2-dependent signaling outcomes in a variety of estrogen target tissues in both normal and pathological states.

To date, two other nuclear receptors have been shown to be targets for acetylation: AR (46) and thyroid hormone receptor (50). Acetylation of AR by p300/CBP and p300/CBP-associated factor decreases corepressor binding, increases coactivator binding, and increases ligand-dependent transactivation (45, 46). In addition, acetylation of AR may also regulate MAPK kinase kinase 1-induced apoptosis (51). Mutation of the AR acetylation sites also inhibits proper trafficking of the receptor, although it is not clear whether this is directly related to impaired acetylation of AR (47). Interestingly, histone deacetylase 1 has been shown to interact with AR in the absence of androgen and dissociate in the presence of androgen, suggesting that reversible acetylation might play a role in regulating activity of AR (51). In contrast to AR, little is known about the functional consequences of thyroid hormone receptor acetylation (50).

Alignment of human ERα with the ERαs from a variety of other species shows that Lys266 and Lys268 are highly conserved from amphibians through mammals (Fig. 5A). In addition, most non-orphan nuclear receptors typically have two or more, and as many as six, lysine residues located in the amino-terminal portion of the CTE, corresponding to the region where Lys266 and Lys268 are located in ERα (Fig. 5B). It is interesting to speculate that this region of nuclear receptors might represent a common target for acetylation. In this regard, note that this region includes the three lysine residues in AR that are acetylated by p300/CBP (Lys630, Lys632, and Lys633) (45–47) (Fig. 5B). Interestingly, human ERβ lacks lysine residues homologous to Lys266 and Lys268 in ERα (Fig. 5B), fitting well with our observation that ERβ is not acetylated by p300 under the same conditions that lead to acetylation of ERα (data not shown). These differences in acetylation by p300 may account for some of the functional differences that have been noted for ERα and ERβ (7–15).

**Acetylation of Lys266 and Lys268 Enhances the DNA Binding and Transactivation Activities of ERα**

Our results demonstrate that acetylation of ERα at Lys266 and Lys268 by p300 enhances the DNA binding activity of ERα (Fig. 7), an effect that appears to be dependent on the neutralization of positive charges at those residues. In fact, substitution of Lys266 and Lys268 for glutamate (i.e. K266/268E), which has a negatively charged side chain, enhances ERα DNA binding activity to an even greater extent (~10-fold more than wild-type ERα; data not shown) than substitution for glutamine (i.e. K266/268Q; ~5-fold; Fig. 7). Because we lack structural information about this region, however, it is not clear whether Lys266 and Lys268 directly contact the DNA or are involved in critical intramolecular interactions that regulate the DNA binding activity of ERα. The former possibility seems less likely because one might expect the loss of positive charge at Lys266 and Lys268 upon acetylation to reduce, not enhance, interactions with negatively charged DNA. Furthermore, Lys266 and Lys268 are located outside of the core DBD in the CTE (Fig. 5A). Results from a recent study suggest that the CTE is required for the binding of ERα to imperfect EREs or half ERE sites and may be a target during the enhancement of ERα DNA binding by high mobility group B-1/2 proteins (17). Acetylation of Lys266 and Lys268 may play a role in modulating the structure of the CTE to enhance the DNA binding activity of ERα. Whether the increase in ERα DNA binding activity observed upon acetylation Lys266 and Lys268 is solely responsible for the concomitant increase in transcriptional activity has not yet been determined.

**A Possible Role for Regulated Posttranslational Modification in Determining the Activity of ERα**

The enzymes that regulate the acetylation state of ERα (*i.e.* the acetylases and deacetylases) are likely to play a key role in modulating the activity of ERα. p300 and its paralog CBP are potent acetylases that play multiple roles in ERα-dependent gene regulation (18, 22). Likewise, deacetylases have also been shown to play important roles in ERα-dependent gene regulation (52–56). Distinguishing the direct effects of these enzymes on ERα acetylation status from their effects on
other transcription-related targets will require further investigation.

Interestingly, resveratrol, an activator of the NAD\textsuperscript{+}-dependent deacetylase SIRT1 (57), has been shown to modulate estrogen-dependent signaling pathways and inhibit estrogen-dependent cell proliferation (58). Although direct effects of resveratrol on the activity of ER\textsubscript{α} are impossible to rule out (59–62), it is interesting to speculate that perhaps some of the antagonistic actions of resveratrol on estrogen signaling relate to its ability to stimulate SIRT1 activity (i.e. resveratrol might enhance the deacetylation of ER\textsubscript{α} by SIRT1, thus reducing ER\textsubscript{α} DNA binding and transcriptional activities). The ability of SIRT1 to deacetylate ER\textsubscript{α} suggests a possible link between nuclear NAD\textsuperscript{+} metabolism, the regulation of ER\textsubscript{α} activity by acetylation, and cell proliferation. If this is the case, the acetylation status of ER\textsubscript{α}, as measured by acetylation-specific antibodies such as the ones described herein, might be a useful additional prognostic indicator for breast cancers.

Also of note with regard to the regulatory aspects of ER\textsubscript{α} posttranslational modification is that Lys266 and Lys268 in ER\textsubscript{α}, which are sites of acetylation, have recently been shown to be targets for SUMOylation as well (63). This suggests an intriguing interplay between these two posttranslational modifications in the regulation of ER\textsubscript{α} activity. As shown previously, multiple covalent posttranslational modifications of a single protein can interact functionally to add additional levels of regulatory control (40, 64–68). This possibility, and the others noted above, will be examined in future studies.

MATERIALS AND METHODS

Chemicals

Acetyl CoA, E2, nicotinamide, NAD\textsuperscript{+}, and TSA were from Sigma-Aldrich (St. Louis, MO). \[\textsuperscript{3}H\]Acetyl CoA was from PerkinElmer Life and Analytical Sciences (Boston, MA).

Synthesis and Purification of Recombinant Proteins

FLAG-tagged wild-type human ER\textsubscript{α} and his\textsubscript{6}-tagged human p300 were expressed in Sf9 cells by using recombinant baculoviruses and purified as described previously (69, 70). Mutant human ER\textsubscript{α} cDNAs, including hER\textsubscript{α}ΔAB(180–595), hER\textsubscript{α}ΔDBD (1–180/269–595), hER\textsubscript{α}L540Q, and various hER lysine point mutants, were generated either by PCR or site-directed mutagenesis. The corresponding FLAG-tagged mutant ER\textsubscript{α} proteins were expressed in Sf9 cells by using recombinant baculoviruses and purified using FLAG M2 affinity chromatography as described for wild-type ER\textsubscript{α}. The his\textsubscript{6}-tagged mouse SIRT1 (also known as Sir2α) expression construct was provided by Shin-ichiro Imai (Washington University, St. Louis, MO). The corresponding protein was expressed in Escherichia coli and purified by standard nickel-nitrilotriacetic acid affinity chromatography. The GST-LBD(282–595) and GST-LBD(282–420) expression plasmids were provided by Benita Katzenellenbogen (University of Illinois, Urbana-Champaign, IL) and Richard Pestell (George-town University, Washington, DC), respectively. The corresp\textsuperscript{oning} GST-fusion proteins were expressed in E. coli and purified by standard glutathione-agarose affinity chromatography. GST-fused SRC2(RID/PID) was expressed in E. coli and purified by glutathione-agarose affinity chromatography as described previously (41). All purified proteins were frozen in aliquots in liquid N\textsubscript{2} and stored at −80 C. Aliquots were analyzed by SDS-PAGE with Coomassie brilliant blue R-250 staining relative to BSA mass standards.

In Vitro ER\textsubscript{α} and Nucleosomal Core Histone Acetylation Assays

ER\textsubscript{α} and nucleosomal core histone acetylation reactions with \[\textsuperscript{3}H\]acetyl CoA were carried out essentially as described previously (41). Briefly, ER\textsubscript{α} was incubated in the presence (Fig. 1B) or absence (all other figures) of salt-dialyzed chromatin, with or without p300, GST-SRC2(RID/PID), E2, and \[\textsuperscript{3}H\]acetyl CoA as indicated in a final volume of 35 μl under reaction conditions described previously (71, 72). The chromatin was prepared by salt dialysis using a plasmid DNA template with four tandem EREs and was purified on sucrose gradients to remove free histones (41, 73). The reactions were incubated at 27 C for 30 min, and aliquots were analyzed by both 10 and 15% SDS-PAGE to resolve ER\textsubscript{α} and core histones, respectively. The proteins in the gels were detected by staining using Coomassie brilliant blue R-250, followed by fluorography. The \[\textsuperscript{3}H\]labeled ER\textsubscript{α} and core histone bands were excised from the gel and quantified by liquid scintillation counting. Acetylation reactions with unlabeled acetyl CoA were carried out under similar reaction conditions; however, the acetylated target proteins were detected by Western blotting with antibodies to acetylated lysine (New England Biolabs, Ipswich, MA) or acetylated ER\textsubscript{α} (see description below). Mock acetylation reactions lacked acetyl CoA or GST-SRC2(RID/PID), as indicated.

In Vitro ER\textsubscript{α} Deacetylation Assays

Purified FLAG-tagged ER\textsubscript{α} was immobilized on FLAG M2-agarose resin and acetylated by p300 in the presence of \[\textsuperscript{3}H\]acetyl CoA as described above to generate \[\textsuperscript{3}H\]acetylated ER\textsubscript{α}. After extensive washing to remove the p300 and \[\textsuperscript{3}H\]acetyl CoA, the ER\textsubscript{α} was eluted by using FLAG peptide, aliquoted, frozen in liquid N\textsubscript{2}, and stored at −80 C until use. For deacetylation reactions, \[\textsuperscript{3}H\]acetylated ER\textsubscript{α} was incubated with HeLa cell nuclear extract or purified SIRT1 in deacetylation buffer [50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 4 mM MgCl\textsubscript{2}] for 40 min at 27 C in the presence or absence of TSA (10 μM), NAD\textsuperscript{+} (400 μM), and nicotinamide (4 mM) in a final volume of 100 μl as indicated. After the reactions were complete, the samples were incubated with FLAG M2-agarose resin for 2 h at 4 C to concentrate the ER\textsubscript{α} protein, followed by extensive washing. The resin was boiled in sodium dodecyl sulfate (SDS) loading solution, and the samples were resolved by SDS-PAGE with subsequent fluorography.

Mass Spectrometric Analysis of Acetylated ER\textsubscript{α}

In vitro acetylation of ER\textsubscript{α} was analyzed by quantitative mass spectrometry (42). ER\textsubscript{α} was acetylated by p300 in the presence of unlabeled acetyl CoA under the conditions described above. The ER\textsubscript{α} was separated from the other proteins in the reaction by SDS-PAGE with subsequent staining using Coomassie brilliant blue R-250. Gel slices containing the ER\textsubscript{α} were excised, treated with iodoacetamide to block oxidation of cysteines, washed, and dehydrated with acetoniitriile. A mixture of 30% deuteroium-acetic anhydride (D6-acetic anhydride) in 100 mM ammonium bicarbonate was added to the gel slices to acetylate all unmodified lysine residues in ER\textsubscript{α} with deuterated acetyl groups. After these chemical modifications, the ER\textsubscript{α} protein was digested with trypsin for 7 h at
37 °C in the gel slices. Because of the complete acetylation of all lysine residues, trypsin cut only after arginine in these reactions. The resulting peptides contained light acetylation on lysines acetylated by p300 and heavy (deuterated) acetylation on all other lysines. This translated into a mass difference of ~3 Da for acetylation visualized by MALDI-QqTOF mass spectrometry (43). The percent acetylation of the chemically identical, isotopically distinct peptides (i.e. heavy vs. light) was calculated directly from ratios of the intensities of the monoisotopic peaks. The specific sites of acetylation in the peptides showing acetyl CoA-dependent acetylation were confirmed by MS/MS analysis using a MALDI-ion trap mass spectrometer (42).

Generation of Acetylated ERα Antibodies

Rabbit antiacetylated Lys266/268 human ERα antiserum (AcK266/268-ERα) was generated by Covance Research Products (Denver, PA) using standard techniques for peptide immunogens. Briefly, a double acetylated peptide spanning amino acids 263 through 273 of human ERα (Arg-Met-Leu-acetyl-Lys-His-acetyl-Lys-Arg-Gln-Arg-Asp) was conjugated to KLH and used as an immunogen in rabbits. Non-acetylation-specific antibodies were depleted from the antiserum by adsorption to a nonacetylated peptide affinity matrix with subsequent collection of the unbound material. The antiserum was screened by ELISA and Western blotting using acetylated and unacetylated purified recombinant human ERα.

In Vivo ERα Acetylation Assays

MDA-MB-231 human breast cancer cells stably expressing FLAG-tagged human ERα (231/ERα cells) (49) were grown in DME/F12 containing 10% charcoal-dextran stripped calf serum. 293T human kidney epithelial cells were grown in DMEM containing 10% FBS and were transfected with a vector reagent (Roche Diagnostics). Each well received the following combination of plasmid DNAs: 1) 5 ng of a pCMV5 vector for the expression of wild-type or mutant ERα, or 5 ng of an empty pCMV5 control vector, 2) 250 ng of an estrogen-responsive luciferase reporter construct containing two EREs upstream of the human pS2 (also known as TFF1) promoter (pGL3-TFF1-pS2-Luc), and 3) 100 ng of pCMVβ, a constitutive β-galactosidase expression vector used for normalization. Twelve hours after transfection, the cells were treated with vehicle or 10 nM E2 for an additional 18 h. Luciferase activity was measured in extracts from the transfected cells using a 96-well plate luminometer (LD400; Beckman Coulter, Fullerton, CA) and normalized to β-galactosidase activity measured in the same extracts using the plate reader. To ensure reproducibility, each assay was run in duplicate, and each experiment was performed at least three times.

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