Vitamin D receptor-interacting protein 150 (DRIP150) has been identified as part of mediator-like complexes that enhance transcriptional activation of the estrogen receptor (ER) and other nuclear receptors (NRs). DRIP150 coactivates ligand-dependent ERα-mediated transactivation in ZR-75 and MDA-MB-231 breast cancer cells transfected with a (luciferase) reporter construct (pEREα) regulated by three tandem estrogen-responsive elements. Coactivation of ERα by DRIP150 in ZR-75 cells was activation function 2-dependent and required an intact helix 12 that typically interacts with LXXLL motifs (NR box) in p160 steroid receptor coactivators. DRIP150 contains C- and N-terminal NR boxes (amino acids 1182–1186 and 69–73, respectively), and deletion analysis of DRIP150 showed that regions containing these sequences were not necessary for coactivation of ERα. Analysis of multiple DRIP150 deletion mutants identified a 23-amino-acid sequence (789–811) required for coactivation activity. Analysis of the protein crystal structure data base identified two regions at amino acids 789–794 and 795–804, which resembled α-helical motifs in Lanuginosa lipase/histamine N-methyltransferase and hepatocyte nuclear factor 1, respectively. By using a squelching assay and specific amino acid point mutations within each α-helix, the NIFSEVRVYN (795–804) region was identified as the critical sequence required for the activity of DRIP150. These results demonstrate that coactivation of ERα by DRIP150 in ZR-75 cells is NR box-independent and requires a novel sequence with putative α-helical structure.

The estrogen receptor (ER) is a ligand-activated transcription factor and a member of the nuclear hormone receptor superfamily (1–5). These proteins exhibit conserved structural domains (A–F) including a central DNA binding domain (DBD) (C), and a ligand binding domain that overlaps activation function 2 (AF2) in the C-terminal (E/F) region. Most nuclear receptors also contain a less conserved N-terminal AF1 (A/B) and a flexible hinge domain (D). The ligand-bound ERα (or ERβ) forms homo- or heterodimers that interact with specific response elements or other DNA-bound nuclear proteins in target gene promoters. A palindromic estrogen-responsive element (ERE) was first identified in the frog vitellogenin A2 gene promoter (6), and other functional consensus and nonconsensus EREs have been characterized in promoters of several E2-responsive genes (7). ERα-mediated transactivation from ERE promoters is dependent on recruitment of an array of nuclear proteins including coactivators that facilitate interactions of ligand-bound ER with the basal transscription machinery (8–14).

Steroid receptor coactivators (SRCs) include SRC1 (NCoA-1), SRC-2 (TIF2/GRIP-1/NCoA-2), SRC-3 (pCIP/ACTR/A1B1), and several related proteins represent one class of coactivators that enhance ERα-mediated transactivation. Interactions of SRCs with ERα and other nuclear receptors is ligand-dependent, and SRCs also exhibit weak histone acetyltransferase and facilitate recruitment of other coregulatory proteins including CBP/300. SRC interactions with ERα and other nuclear receptors are dependent on one or more LXXLL motifs that interact with helix 12 in the AF2 region (15–19). Several other classes of nuclear receptor coactivators have now been identified, and their interactions with ERα and other receptors can be LXXLL-dependent or -independent. For example, cavelolin-1 interacts with ERα and coactivates ERα-mediated transactivation, and the scaffolding domain of cavelolin-1 is required for this response (20, 21).

Another important class of coactivators resembles the mammalian Mediator complex, and these have been termed vitamin D-interacting proteins (DRIPs) or thyroid hormone-associated proteins (TRAPs) (22–25). Many of the DRIPs/TRAPs have also been identified in other large coregulatory complexes such as NAT, ARC, CRSP, and SMCC (26–29). DRIP205 and TRAP220 are identical to peroxisome proliferator-activated receptor-binding protein (30), and several studies show that interaction of ERα and nuclear receptors with the DRIP/TRAP complex occurs via DRIP205/TRAP220, which anchors the complex to the receptor.

DRIP205 contains two LXXLL motifs that are required for ligand-dependent interactions with nuclear receptors including ERα and ERβ (31–36). There is also evidence suggesting that DRIP205, other mediator complex proteins, and p160 coactivators/p300 coordinately interact to enhance ERα-dependent transactivation (37, 38). For example, a recent study analyzed ERα-coactivator interactions on the pS2 gene promoter by chromatin immunoprecipitation assays and showed a cyclic association and
dissociation of DRIP205 and p160 SRs but in opposite phases (38). This is consistent with different temporal patterns of coactivator-receptor interactions that may cooperatively enhance nuclear hormone receptor-dependent transactivation. It has also been reported that DRIP150 interacts with ER and other nuclear receptors in vitro (37–39). Moreover, analysis of coactivator assembly on the pS2 gene promoter by chromatin immunoprecipitation shows that at some time points, both ERα and DRIP150 are cross-linked to the promoter in the absence of DRIP205 (38). This study investigates coactivation of ERα by DRIP150 in ZR-75 breast cancer cells transfected with a construct containing three tandem EREs (pERE3). DRIP150 coactivates ERα in ZR-75 cells, and coactivation is AP2-dependent. However, studies with mutant DRIP150 constructs show that coactivation of ERα is independent of the two LXXLL in NR box motifs present in the C- and N-terminal regions of DRIP150. Coactivation of ERα required a unique 23-amino acid sequence (amino acids 789–811) in DRIP150, and mutational analysis has further identified a putative helical region at amino acids 795–804 (NIFSEVRVNY) that is required for hormone-induced transactivation.

MATERIALS AND METHODS

Cell Lines, Chemicals, and Biochemicals—The ZR-75 human breast cancer cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA), and cells were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (FBS) (Summit Biotechnology, Fort Collins, CO). medium was further supplemented with sodium bicarbonate, glucose, Hepes, sodium pyruvate, and antibiotics/antimycotic solution (Sigma). MDA-MB-231 cells were obtained from the ATCC and maintained in Dulbecco’s modified Eagle’s medium/F-12 supplemented with charcoal-stripped FBS. Cells were maintained at 37 °C in a humidified CO2/air (5:95) mixture. Phenol-free Dulbecco’s modified Eagle’s medium/F-12 plus 2.5% charcoal-stripped FBS for 36 h. Cells were transfected with the calcium phosphate method with 1 μg of pERE3 reporter and 0.25 μg of a cytomegalovirus β-galactosidase expression plasmid, the appropriate ERα expression plasmid, and the appropriate DRIP150 expression plasmid. After 6–8 h, cells were harvested by scraping the plates in 100 μl of 1× lysis buffer (Promega). Thirty five μl of the cell lysate were used for performing luciferase assays on a Lumicount Luminometer (Packard Instrument Co.). Thirty five μl of the cell lysate was used for determining β-galactosidase activity on a luminometer. Normalized luciferase values were calculated by dividing the luciferase by the β-galactosidase activities for a given sample. Results were expressed as means ± S.E. for at least three experiments for each treatment group and were compared with the MeSO control group (arbitrarily set at 1) for each set of experiments.

Gel Electrophoretic Mobility Shift Assays—Five picomoles of synthesized ERE was labeled at the 5’ end using T4-polynucleotide kinase and [γ-32P]ATP. Plasmids containing the DRIP150, m1, m2, and m3 cDNAs were used in vitro transcript and translate the corresponding protein in a rabbit reticulocyte lysate system (Promega). Three μl of recombinant human ERα (500 fmol) was mixed with 3 μl of bovine serum albumin (500 ng/μl), 2 μl of polyclonal antibody (1 μg/ml), 5 μl of 5× binding buffer (20 mM Heps, 5% glycerol, 100 mM KCl, 5 mM MgCl2, 0.5 mM dithiothreitol, 1 mM EDTA), and 1 μl of E2 (3,5 × 10−7 M) to give an final concentration of 2.5 × 10−6 M E2, and incubated on ice for 15 min. In vitro translated DRIP150, m1, m2 or m3 were then added to the above mixture and incubated on ice for 5 min. To balance the volume, in vitro translated pcDNA3 was also added. For supershift experiments, 2 μl of normal IgG or ER antibody was added to the mixture after 5 min and then incubated on ice for an additional 5–10 min, and 5 μl of 32P-labeled ERE probe (120,000 cpm) was added to the reaction mixture, giving a final volume of 25 μl. The mixture was incubated at 20 °C for 15 min. Samples were resolved by polyacrylamide gel (10%) electrophoresis at 10 V/cm in 0.09 M Tris, 0.09 M borate, 2× EDTA, pH 8.3, for 2.5 h. The gel was dried and exposed to a phosphor screen for 12 h, and protein-DNA binding was visualized by autoradiography using a Storm PhosphorImager (Amer sham Biosciences).

Coimmunoprecipitation Assays—Two hundred μl of reticulocyte lysate was mixed with 40 μl of protein G plus-agarose and 17 μl of ER antibody and shaken for 1 h at 4 °C to preclude ER expression in the

primer CTG TGG ATC ATG ACC AGC TTC CAC CAC AAA and lower primer TCA TGG TGC ACT CAG ACT GTG GCA GGA AAA CC. After PCR amplification, the ER-cDNA fragment was digested with BamHl and Sacl and cloned into mPF10 digested with BamHl and Sacl to give pM-ER. The 17m5-GA4-Luc plasmid containing five copies of the yeast GAL4 recognition motif linked to a luciferase reporter gene was provided by Dr. Patrick Balaguer (INSERM 458, Montpellier, France) and Tim Zacharewski (Michigan State University, East Lansing, MI).

Cloning of DRIP150 Mutants—The A1145–1454 m1(mutant)1 of DRIP150 was generated by KpnI/XhoI digestion of plasmid SDNA and Sacl and cloned into mPF10 digested, and the fragments were run on 1% agarose gel, and 3.5-kb fraction was eluted and ligated with KpnI/Xhol cut pcDNA3 vector. Except for m1, all other clones expressing DRIP150 mutants were generated by PCR amplification, and primers used for preparing DRIP150 mutants and GAL4DBD fusion proteins are summarized in Table I and Table II. Xpress-tagged m2 and m3 DRIP150 mutants were generated by cloning DRIP150 fragments into Xpress-tagged HisA-pcDNA3.1 vector. mPDF150 was generated by inserting DRIP150 into pM vector. pM23 and related point mutants were generated by inserting DRIP150 23 aa (789–811 region) and the 23-aa region with the mutated DRIP150 aa 792 (Ala→Pro) or double mutant into the pM vector.

After PCR amplification, cDNA fragments of m2 and m3 were digested with KpnI/XhoI and cloned into pcDNA3.1 vector. Recombinant human ERα protein was obtained from Panvera (Madison, WI), and all other chemicals and biochemicals were obtained from commercial sources at the highest quality available.

Oligo(dT)20 Labeled Oligonucleotides and Plasmids—The consensus estrogen-response element (ERE) probe used in gel mobility shift assays was synthesized by the Gene Technologies Laboratory (College Station, TX), and the sequence was 5’-GTC CAA AGT CAG TGC ACA TTG AAG TT-3’. ERE expression plasmid was kindly provided by Dr. Ming Jer Tsai (Baylor College of Medicine, Houston, TX). Expression plasmids for ER mutants with deletion of amino acids 1–178 (HE19) and TAF1 containing D536N, E542K, and D545N mutations were kindly provided by Dr. Pierre Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch, France) and Dr. Donald McDonnell (Duke University, Durham, NC), respectively. cDNA encoding DRIP150 was kindly provided by Dr. Leonard P. Freedman (Merck). The expression plasmid for the GR1 NR box polypeptide GAL4 fusion protein was also kindly provided by Dr. Donald McDonnell (Duke University). The expression plasmid for the AF1 polypeptide was generated in that laboratory by cloning amino acids 1–180 of ERα into NheI/EcoRI site of pcDNA3 vector. pcDNA3-His-LucZ was purchased from Invitrogen. The pERE3 reporter containing three consensus ERE sites linked to a luciferase gene was created by cloning an oligonucleotide with three ERE elements into BamHI-HindIII cut pXP2 plasmid (40). ERα-GAL4 fusion protein was constructed as follows. First, the GAL4DBD fusion protein vector pCMV2p1-C (Clontech) was digested with BamHI and HindIII, and the oligonucleotide sequence CAT CGG TCT CGT CAG ACG TCG ACA was inserted into this digested vector. This oligonucleotide was added to create more space between restriction enzymes BamHI and SalI in the polylinker of vector pM, providing a more efficient digestion of these two enzymes when cut simultaneously. This new vector, pM (-10), was then used for construction of the pM-ER plasmid. Primers used for preparing GAL4DBD fusion protein with ER were upper

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min. Ten H9262 at 4 °C with shaking every 30 min. For samples not containing 35S-agarose (1:2 ratio) and added to the above mixture and incubated for 3 h mixed with 2 reticulocyte. After 1 h, the mixture was centrifuged at 1,500 g and pcDNA3.0. DRIP150, ER, and pcDNA3.0 were mixed with 20 μl of in vitro translated 35S-DRIP150 was translated Xpress-tagged m2 and m11, Xpress-tagged DRIP150 m2 and DRIP150 m11 were translated 35S-DRIP150 was translated with 1 μl of ERα antibody was then mixed with protein G plus-agarose (1:2 ratio) and added to the above mixture and incubated for 3 h at 4 °C with shaking every 30 min. For samples not containing 35S-DRIP150, only 35S-labeled ER (2 μl) was mixed with ER antibody-protein G plus-agarose mixture and incubated for 3 h at 4 °C as described above. PBS (1 ml) was then added to each sample, shaken for 30 s, and then centrifuged at 1,500 x g for 5 min. After centrifugation, the supernatant was discarded, and the pelleted fraction (100 μl) was mixed with 20 μl of 1 x sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromphenol blue, 10% glycerol, and 100 mM diethiothreitol) containing β-mercaptoethanol. The sample was then boiled for 5 min, loaded onto SDS-polyacrylamide gel, and run at 150 V for 4 h. The gel was dried and exposed to a phosphoscreen for 3 days, and proteins were visualized by autoradiography using a Storm PhosphorImager (Amer sham Biosciences).

Western Immunoblot Assays—COS-7 cells (from ATCC) were seeded in 6-well plates at a concentration of 200,000 cells/well in phenol-free Dulbecco’s modified Eagle’s medium/F-12 with 2.5% charcoal-stripped FBS. After 24 h, the media were removed, and serum and antibiotic-free, phenol-free Dulbecco’s modified Eagle’s medium/F-12 was added to the wells. X-press-tagged DRIP150 m2 and DRIP150 m11 were transferred using the Lipofectamine transfection method (Invitrogen). After 6 h, the media were removed, and phenol-free Dulbecco’s modified Eagle’s medium/F-12 with 2.5% charcoal-stripped FBS was added, and cells were incubated for 36 h. Cells were then harvested in lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 10 μg/ml aprotinin, 50 μM phenylmethylsulfonyl fluoride, 50 mM sodium orthovanadate), placed on a rocker at 4 °C to extract soluble protein, and centrifuged at 14,000 g for 10 min at 4 °C. Protein was quantitated, and an equal amount of protein (150 μg) was diluted with loading buffer, boiled, and loaded on 10% SDS-polyacrylamide gel. Samples were electrophoresed at 150–180 V for 3–4 h. For samples containing in vitro translated Xpress-tagged m2 and m11, Xpress-tagged DRIP150 m2 and DRIP150 m11 were translated in vitro in a rabbit reticulocyte lysate system (Promega), diluted with loading buffer, boiled, loaded on 10% SDS-polyacrylamide gel, and electrophoresed at 150–180 V for 3–4 h. The separated proteins were transferred (in a buffer containing 48 mM Tris-HCl, 29 mM glycine, and 0.025% SDS) to polyvinylidene difluoride membrane (Bio-Rad). Specific proteins were detected by incubation with mouse monoclonal anti-Xpress antibody (1:5000 dilution) for 4 h, rinsed with distilled water (three times), followed by blotting with horseradish peroxidase-conjugated anti-mouse secondary antibody (1: 5000 dilution) for 1.5 h. The membrane was then washed with PBS/ Tween 20 (0.05%), and blots were exposed to chemiluminescent substrate (ECL) (PerkinElmer Life Sciences) and placed on Kodak X-Omat film. The detected bands were scanned using a Storm PhosphorImager (Amer sham Biosciences).

Statistical Analysis—Statistical differences between different treat-
ment groups were determined using Student’s t test or analysis of variance (Fisher’s protected Least Significant Difference), and the levels of significance were noted (p < 0.05). The results were expressed as mean ± S.E. for at least three replicate determinations for each experiment.

RESULTS

DRIP150 Coactivation of ERα—DRIP150 is a member of the mediator complex of proteins, and this study investigates coactivation of ERα by DRIP150 in ZR-75 cells transfected with pERE3, ERα expression plasmid (50 ng), and DRIP150 expression plasmid (2.5–7.5 ng) treated with MeSO or 10 nM E2, and luciferase activity was determined as described under “Materials and Methods.” Significant (p < 0.05) coactivation of E2-induced activity is indicated by an asterisk, and results are expressed as means ± S.E. for at least three separate determinations for each treatment group. Coactivation was also observed using 10, 25, or 150 ng of ERα expression plasmid (data not shown). Significant coactivation by DRIP150 (or mutants) in this study represents an increase in the fold induction compared with that observed for E2 alone. Hormone responsiveness was not observed in the absence of cotransfected ERα. B, coactivation of pM-ERα by DRIP150. ZR-75 cells were transfected with pM (empty vector), pM-ERα (50 ng), or DRIP150 (200 or 400 ng), treated with E2 or MeSO, and luciferase activity determined as described under “Materials and Methods.” Significant (p < 0.05) induction by E2 (*) and coactivation by DRIP150 (**) is indicated. C, coactivation of ERα in MDA-MB-231 cells. Cells were treated as described in A, and significant (p < 0.05) coactivation by DRIP150 is indicated (**). Coactivation of HE19 (D) and TAF1 (E) by DRIP150. ZR-75 cells were treated with 10 nM E2 or MeSO, transfected with the indicated amounts of plasmids, and luciferase activity determined as described under “Materials and Methods.” Significant (p < 0.05) coactivation is indicated (**). SNURF coactivation of ERα (D) and DRIP150 coactivation of ERα (E) serve as positive controls for these experiments.

FIG. 1. Coactivation of wild-type and variant ERα by DRIP150 in ZR-75 and MDA-MB-231 cells. A, coactivation of ERα. ZR-75 cells were transfected with pERE3, ERα expression plasmid (50 ng), and DRIP150 expression plasmid (2.5–7.5 ng) treated with MeSO or 10 nM E2, and luciferase activity was determined as described under “Materials and Methods.” Significant (p < 0.05) coactivation of E2-induced activity is indicated by an asterisk, and results are expressed as means ± S.E. for at least three replicate determinations for each treatment group. Coactivation was also observed using 10, 25, or 150 ng of ERα expression plasmid (data not shown). Significant coactivation by DRIP150 (or mutants) in this study represents an increase in the fold induction compared with that observed for E2 alone. Hormone responsiveness was not observed in the absence of cotransfected ERα. B, coactivation of pM-ERα by DRIP150. ZR-75 cells were transfected with pM (empty vector), pM-ERα (50 ng), or DRIP150 (200 or 400 ng), treated with E2 or MeSO, and luciferase activity determined as described under “Materials and Methods.” Significant (p < 0.05) induction by E2 (*) and coactivation by DRIP150 (**) is indicated. C, coactivation of ERα in MDA-MB-231 cells. Cells were treated as described in A, and significant (p < 0.05) coactivation by DRIP150 is indicated (**). Coactivation of HE19 (D) and TAF1 (E) by DRIP150. ZR-75 cells were treated with 10 nM E2 or MeSO, transfected with the indicated amounts of plasmids, and luciferase activity determined as described under “Materials and Methods.” Significant (p < 0.05) coactivation is indicated (**). SNURF coactivation of ERα (D) and DRIP150 coactivation of ERα (E) serve as positive controls for these experiments.
Coactivation of ERα by DRIP150 in ZR-75 Cells

described in Fig. 1A, it was also shown that DRIP150 coactivates ERα-mediated transactivation in ER-negative MDA-MB-231 breast cancer cells transfected with pERE3, and the fold enhancement was >5 (Fig. 1C). Thus, comparable coactivation results were obtained in both ER-positive (ZR-75) and ER-negative (MDA-MB-231) breast cancer cell lines.

ERα contains two major activation domains, and we therefore investigated the coactivation activity of DRIP150 in cells transfected with HE19 (deletion of AF1) and ERα-TAF1, which contains three amino acid mutations in helix 12 (D538N, E542Q, and D545N), which inactivates AF2 (41). The results in Fig. 1D demonstrate that in ZR-75 cells transfected with pERE3 and HE19, treatment with E2 increased (>50%) luciferase activity, and this response was further enhanced by cotransfection with DRIP150. As a positive control for this experiment, we also observed coactivation of HE19 by the RING finger protein SNURF as reported previously (40). A higher level of coactivation by SNURF was observed, and this may be due, in part, to the cooperative coactivation of HE19 by SNURF and TATA-binding protein (40). E2 induced luciferase activity in ZR-75 cells transfected with ERα-TAF1; however, this response was not enhanced by DRIP150 (Fig. 1E), whereas in the same experiment, DRIP150 coactivated wild-type ERα (positive control).

These results suggest that DRIP150 primarily coactivates ERα through direct or indirect interactions with the AF2 domain, and this was further investigated by competition (squelching) experiments with NR box and AF1 proteins (AF1-p). The results in Fig. 2A demonstrate that increasing amounts of the 2XF6 peptide (42), which contains two NR boxes fused to the yeast GAL4-DBD, significantly decreased DRIP150 coactivation of ERα in ZR-75 cells. In contrast, transfection with the AF1 protein that contains amino acids 1–182 from ERα did not significantly decrease (or squelch) DRIP150 coactivation of ERα (Fig. 2B), whereas this protein inhibited AF1-dependent activation of GC-rich promoter constructs by ERαSp1 (43). These results suggest that coactivation of ERα by DRIP150 is primarily AF2-dependent.

Interactions of ERα and DRIP150—Kang and coworkers (44) reported that in nuclear extracts containing DRIP complex proteins, both wild-type and the ligand binding domain of ERα interacted with DRIP150 in pulldown assays; however, DRIP150-ERα interactions were not observed unless DRIP205 was also expressed. Results illustrated in Fig. 3A used in vitro translated and radiolabeled 35S-ER and 35S-DRIP150 in coimmunoprecipitation experiments. ERα antibodies coimmunoprecipitate ERα alone and in combination with DRIP150, indicating that both proteins directly interact. We also investigated direct interactions of DRIP150 and ERα in a mammalian two-hybrid assay in MDA-MB-231 cells transfected with pM-DRIP150 plus VP-ERα expression plasmids (Fig. 3B). E2 had no effect in cells transfected with pM/VP (empty vectors), pM/VP-ERα, or pM-DRIP150/VP-ERα; however, a significant induction of luciferase activity by E2 was observed after cotransfection with pM-DRIP150 and VP-ERα. This experiment was also carried out as a mammalian one-hybrid assay in MDA-MB-231 cells transfected with pM-DRIP150 and ERα, and the results showed that ERα-enhanced transcription was ligand-dependent (Fig. 3C). These results paralleled the complementary coactivation of pM-ERα by DRIP150 (Fig. 1B) and confirmed interactions between ERα and DRIP150. Interactions of the in vitro translated proteins (unlabeled) were also investigated in gel mobility shift assays using 32P-ERE (Fig. 3D). Incubation of ERα and 32P-ERE gave a retarded band (Fig. 3D, 1st lane), and coincubation with increasing amounts of DRIP150 increased intensity of the retarded band (2nd to 4th lanes), but a super-
shifted ternary complex was not observed. DRIP150 alone did not form a complex with $^{32}$P-ERE (Fig. 3D, 5th lane), and the DRIP150-enhanced complex was supershifted by ERα/H9251 antibodies (7th lane) but not by nonspecific IgG (6th lane). Thus, DRIP150 enhanced ER-ERE complex formation, and similar observations have been reported previously for other transcription factors (including ERα) in gel mobility shift assays where two interacting proteins did not form a ternary complex with DNA; however, protein-DNA binding of one protein was enhanced by the other protein (45–48).

Coactivation of ERα by Mutant DRIP150 Constructs—Wild-type DRIP150 contains 1454 amino acids (aa) with two putative NR boxes at amino acids 1182–1186 and 69–73. Fig. 4A summarizes the effects of wild-type DRIP150 and mutants containing deletions of aa 1145–1454 (DRIP150m1), 789–1454 (DRIP150 m2), and 325–1454 (DRIP150m3) on coactivation of ERα in ZR-75 cells transfected with pERE. The results show that DRIP150m1 was the only one of these deletion mutants that coactivated ERα, suggesting that the C-terminal NR box was not required for coactivation, and the N-terminal NR box

FIG. 3. DRIP150-ERα interactions. A, coimmunoprecipitation. In vitro expression of $^{35}$S-labeled DRIP150 or ERα were immunoprecipitated by ERα antibodies as described under “Materials and Methods.” $^{32}$P-pcDNA3 (empty vector) served as a control. B, mammalian two-hybrid assay. MDA-MB-231 cells were transfected with various constructs and treated with MeSO or E2, and luciferase activity was determined as described under “Materials and Methods.” Results are expressed as means ± S.E. for three separate determinations for each treatment group, and significant ($p < 0.05$) induction by E2 is indicated (*). C, mammalian one-hybrid assay. MDA-MB-231 cells were transfected with various constructs, treated with MeSO or E2, and analyzed essentially as described in B. Significant ($p < 0.05$) estrogen-dependent coactivation of pM-DRIP150 by ERα is indicated (*). D, gel mobility shift assays. $^{32}$P-ERE was incubated with ERα, in vitro expressed DRIP150 in the presence or absence of IgG (nonspecific) or ERα antibodies, and examined in a gel mobility shift assay as described under “Materials and Methods.” In a separate experiment, excess unlabeled ERE also decreased intensity of the specifically bound retarded band (Bound DNA).
was not sufficient for coactivation. The results also indicate that the 789–1144 aa are required for coactivation of ERα/H9251 on an ERE promoter. The results in Fig. 4A were obtained using 2.5 ng of wild-type/mutant DRIP150 expression plasmid; however, similar results were observed for DRIP150 mutants over a range of plasmid concentrations in separate experiments (data not shown). Coactivation of ERα was further investigated with a series of DRIP150 mutants with deletions.
Coactivation of ERα by DRIP150 in ZR-75 Cells

![Graph](image)

**Fig. 5. Coactivation and squelching by DRIP150m8.** A, coactivation of ERα. ZR-75 cells were treated with Me₆SO or 10 nM E₂ transfected with pERE₉ ERα (50 ng), and different amounts of DRIP150m8 expression plasmid, and luciferase activity was determined as described under "Materials and Methods." Results of all experiments illustrated in this figure are means ± S.E. for at least three experiments for each treatment group, and significant (p < 0.05) coactivation (*) and inhibition of coactivation (**) are indicated. B, DRIP150m8 squelching of ERα-mediated transactivation. ZR-75 cells were treated with Me₆SO or 10 nM E₂ and transfected with pERE₉ ERα (10 ng), DRIP150 (5 ng), and different amounts of DRIP150m8 expression plasmid, and luciferase activity was determined as described under "Materials and Methods." Significant (p < 0.05) coactivation by wild-type DRIP150 (*), and inhibition by cotransfection with DRIP150m8 (**) are indicated. C, DRIP150m8 squelching of HE19. This experiment was carried out as described in B, except that HE19 expression plasmid was used.

and B, demonstrate that the C-terminal NR box of DRIP150 is not required for coactivation and that amino acids 789–864 are necessary for coactivation.

An additional series of DRIP150 mutants containing deletions of 850–1454 (DRIP150m9), 827–1454 (DRIP150m10), 812–1454 (DRIP150m11), and 1–77/865–1454 aa (DRIP150m12) were also investigated as coactivators of ERα. The activities of these constructs were determined in separate experiments where there was some variability in the fold induction by E₂ and the amount of mutant DRIP150 expression plasmid required to give maximal coactivation. Therefore, data obtained for these constructs are reported as fold enhancement of coactivation compared with cells treated with E₂ alone (no coactivation). The results showed that all the DRIP150 deletion constructs coactivated ERα. The deletion of the N-terminal NR box (DRIP150m12) did not result in loss of coactivation, showing that this motif was not necessary for DRIP150 coactivation of ERα. Thus, results of deletion analysis of DRIP150 indicate that the 23 amino acids between aa 789 and 811 were required for coactivation of ERα in ZR-75 cells.

We also investigated interactions of ERα and ³²P-ERE in the presence or absence of *in vitro* expressed DRIP150 expression plasmids (Fig. 4C). The ERα-ERE retarded band (first lane) intensity was enhanced after coincubation with wild-type DRIP150 (second lane) and deletion mutants m1, m2, and m3 (third to fifth lanes). Wild-type DRIP150 alone did not bind ³²P-ERE (fifth lane), and ERα antibodies (seventh lane) but not IgG (sixth lane) supershifted the retarded band as indicated in Fig. 3B. The enhanced ERα-ERE retarded band intensity was observed after coincubation not only with wild-type DRIP150 and mutant m1, which coactivate ERα, but also with mutants m2 and m3 that are inactive as coactivators. These results suggest that this response may reflect interactions of DRIP150 mutants with ERα *in vitro*, but these interactions did not predict their activities as coactivators of ERα. DRIP150m2 and DRIP150m11 have similar molecular weights as illustrated in Fig. 4E in which *in vitro* expressed DRIP150 mutants were analyzed by SDS-PAGE and Western blot analysis (lanes 1 and 2). These proteins were also observed in whole cell lysates after transfection (Fig. 4E, lanes 3 and 4) in COS-7 cells.

Coactivation/Squelching by DRIP150 Coactivation Peptide—DRIP150m8 plasmid expresses amino acids 755–885, which encompass the region of DRIP150 required for coactivation of ERα. DRIP150 and other deletion mutants also coactivate ERα, demonstrating that pM23 and other constructs coactivated ERα (Fig. 5C). Moreover, pM23 also inhibits DRIP150 coactivation of ERα and cotransfection with DRIP150m8 inhibits or squelches the coactivation response. pM23 contains the minimal sequence of DRIP150 (aa 789–811, DIPAHLNIF-SERVVNYRKILC) necessary for coactivation of ERα, and this peptide is fused to the DBD of the yeast GAL4 protein (Fig. 6A). Transfection of ZR-75 cells with pERE₉ and different amounts of pM23 expression plasmid showed that this chimeric protein coactivates ERα and then squelches this response with increasing amounts of transfected plasmid (Fig. 6B). This parallels a similar coactivation/squelching response observed for DRIP150m8 (Fig. 5A). Moreover, pM23 also inhibits DRIP150 coactivation of ERα (Fig. 6C), demonstrating that pM23 and DRIP150m8 exhibit comparable coactivation of ERα at low concentrations but also squelch transactivation (at higher concentrations) and inhibit DRIP150 coactivation of ERα. We also examined the protein crystal structure data base for similarities between the DRIP150 amino acid sequence 789–811 with
other proteins. The first six residues DIPAHL fold into an α-helix when they occur in Lanuginosa lipase (49) and histamine N-methyltransferase (50). There was also homology between residues 7 and 16 (NIFSEVRVYN) of the DRIP150 23-amino acid sequence and an α-helical region in hepatocyte nuclear factor 1 (HNF1; NLVTEVRVYN) (51). Results in Fig. 7, A and B, summarize squelching experiments with pM23R801P and pM23A792P, which express the GAL4–23-amino acid fusion protein with mutations at amino acids 801 (Arg → Pro) and 792 (Ala → Pro). Proline residues were inserted to disrupt α-helical structure. The results show that pM23R801P did not squelch DRIP150 coactivation of ERα (Fig. 7A), whereas pM23A792P exhibited wild-type (pM23) activity and squelched DRIP150 coactivation of ERα (Fig. 7B). Squelching of DRIP150 coactivation of ERα was also not observed using the double mutant pM23A792P/R801P (Fig. 7C). These data suggest that the sequence at amino acids 795–804 in pMDRIP150, which resembles an α-helical motif in HNF-1, is an important structural feature of DRIP150 required for coactivation of ERα. These data suggest that in addition to LXXLL motifs, other helical sequences in coactivators can play a role in coactivation of ERα and possible other nuclear receptors in breast cancer cells.

**DISCUSSION**

Several nuclear coregulatory complexes that associate with transcription factors and potentiate RNA polymerase II transcription have been identified, and many of their individual subunits are identical (23–29). The functions of the DRIP, TRAP, NAT, ARC, and CRISP coregulatory complexes are similar to that described for Mediator complexes initially purified from yeast. Interactions of these coregulatory complexes with NRs, including ERα and ERβ, have been investigated, and there is evidence in some cell lines that DRIP205 anchors the protein complex to NRs (23, 25, 31, 44). Several reports have investigated DRIP205 coactivation of NRs including both ERα and ERβ. DRIP205-dependent coactivation of ER depends on both cell context and ER subtype, and the NR boxes of DRIP205 are required for coactivation (33–38). Both DRIP205 and DRIP150 also directly interact with ERα/ERβ, and other stud-
ies confirm that DRIP150 interacts with the glucocorticoid and androgen receptors (44, 52, 53).

Previous studies (52) indicate that DRIP150 coactivated glucocorticoid receptor-mediated transactivation, and this response was AF1-dependent; however, coactivation of ERα by DRIP150 has not been extensively investigated. DRIP150 co-activates ERα in MDA-MB-231 and ZR-75 cells transfected with pEREα, and similar enhancement of transactivation was observed in a mammalian one-hybrid assay in cells transfected with pEREα/GAL4-luc (Fig. 1B). E2-dependent coactivation of DRIP150 by ERα has also been observed (Fig. 3C). DRIP150 also coactivated HE19 but not ERα-TAF1 in ZR-75 cells, suggesting that the AF1 domain of ERα was not necessary for coactivation and that an intact helix 12 was required.

These results are in contrast with the reported AF1-dependent coactivation of glucocorticoid receptor by DRIP150 (53) but are comparable with previous studies (15–19) in several different cell lines showing that helix 12 is a critical surface of ERα that interacts with NR boxes of p160 coactivators. The importance of the AF2 region of ERα for coactivation by DRIP150 was supported by the inhibitory effects or squelching of enhanced transactivation in ZR-75 cells transfected with an NR box expression plasmid (Fig. 2A). This construct contains two copies of the GRIP1 NR box and inhibits ERα-mediated transactivation (42). In contrast, overexpression of an AF1 peptide (aa 1–182 of ERα) (Fig. 2A) did not affect coactivation of ERα by DRIP150, confirming the important role of AF2 of ERα.

DRIP150 contains two LXXLL NR box motifs in the N-
Coactivation of ERα by DRIP150 in ZR-75 Cells

(69–73) and C-terminal (1182–1186) regions. Their role in co-activation of ERα by DRIP150 has not been determined; however, some previous studies show that NR boxes are critical regions for the coactivation of NRs by DRIP205 (32–34). Research in this laboratory has demonstrated recently (54) that coactivation of ERα by DRIP205 in ZR-75 cells is complex, and coactivation does not require the NR boxes. Deletion analysis of DRIP150 (Figs. 4 and 5) shows that coactivation of ERα by DRIP150 deletion variants was NR box-independent, and a 23-amino acid sequence (aa 789–811) was identified as an essential region for DRIP150 coactivation of ERα (Fig. 4). Wild-type DRIP150 coimmunoprecipitates ERα (Fig. 3A) as reported previously (44), and ligand-dependent DRIP150-ERα interactions were observed in a mammalian two-hybrid assay (Fig. 3B). However, in gel mobility shift assays DRIP150 does not form a DRIP150-ERα-ERE ternary complex but enhances the ERE-ERE retarded band intensity (Fig. 3D). The failure to observe a supershifted ternary complex is not unprecedented because previous studies report that ERα enhances Sp1/Sp3 DNA binding (45, 55), cyclin D1 enhances ERα DNA binding, and human T-cell lymphotropic virus type I transcriptional activator (Tax) enhances CREB DNA binding and binding of other transcription factors in gel mobility shift assays (45–47). Most interestingly, the results also show that DRIP150 and DRIP150m1, which coactivate ERα, also enhance the ERα-ERE retarded band. However, mutants that are inactive as coactivators (DRIP150m2 and DRIP150m3) exhibit comparable activity in the gel shift assay (Fig. 4D). This suggests that enhancement of ERα-ERE binding by DRIP150 variants is not predictive for coactivation of ERα-mediated transactivation, which requires the 23-amino acid 789–811 sequence.

We have further investigated the role of the DRIP150 “coactivation sequence” in hormone-induced transactivation using DRIP150m8 that contains amino acids 755–885 and pM23, which contains DRIP150 amino acids 789–811 fused to the yeast GAL4-DBD. Transfection of either protein gave a biphasic response typical of many coactivators in which low concentrations resulted in coactivation of ERα and higher amounts of transfected plasmids subsequently decreased or squelched transactivation (Fig. 5). pM23 and/or DRIP150m8 also inhibit wild-type and mutant DRIP150 coactivation of ERα or HE19 (Fig. 5), and these responses are similar to those observed for NR box peptides containing LXLL sequences (42). Our results confirm that DRIP150 interacts with ERα as reported previously (44) and coactivates ERα in ZR-75 (and MDA-MB-231) cells transfected with pEREα. The coactivator activity of DRIP150 alone in ZR-75 cells contrasts with previous reports showing that ligand-dependent recruitment of mediator complex proteins to ERα and other nuclear receptors requires DRIP205 as an anchor component for complex-receptor interactions (23, 25, 31, 44). However, other reports show that DRIP150 alone interacts with nuclear receptors (52, 53), and this has now been observed for ERα (Fig. 3A). A recent study (56) also reported isolation of a transcriptionally active coactivator CRSP-mediator complex that contained CRSP150/DRIP150 but not DRIP205/Med220 (or Med70), suggesting that DRIP205 is not always required for a functional mediator coactivator complex. This is also supported, in part, by chromat immunoprecipitation studies on the time-dependent recruitment of coactivators, such as SRCs and DRIPs, to the ERE of the pS2 gene promoter in MCF-7 cells (38). The results showed that at some time points, DRIP150 was associated with the pS2 promoter in the absence of DRIP205, suggesting a DRIP150-independent role for DRIP150 as a coactivator of ERα, and this is consistent with the results of this study.

DRIP150 coactivation of ERα is independent of the two NR boxes and requires a 23-amino acid sequence DIPAHLNFSEVRVYNYRLKILC at 789–811 (Figs. 4 and 5). By using the protein crystal structure data base, there was not a good match between the 23-amino acid DRIP150 sequence and other known crystalline proteins; however, the first six residues DIPAHL fold into an α-helix when they occur in Lanuginosa lipase and histamine N-methyltransferase (49, 50). Amino acids 795–804 in DRIP150 are homologous to amino acids 759–766 in the first helical component of CRSP150/H9251 (51). pM23 efficiently squelches DRIP150 coactivation of ERα (Fig. 6C), and we used this assay to identify the function of the two helical components within the 789–811-amino acid region of DRIP150. Results in Fig. 7 show that pM23A792P/R801P exhibited wild-type (pM23) squelching activity, whereas pM23R801P and pM23A792P/R801P (double mutant) did not squelch coactivation of ERα by DRIP150. These data suggest that the α-helical structure within the NIFSEVRVYN (amino acids 795–804) sequence is required for the activity of DRIP150 as a coactivator of ERα.

In summary, results of this study uniquely identify a novel sequence in DRIP150 required for coactivation of ERα and demonstrate that LXXLL boxes in DRIP150 are not required for enhancement of ERα-dependent transactivation. However, amino acids 795–804 within the critical 789–811 region of DRIP150 required for coactivation of ERα are homologous with an α-helical region in hepatocyte nuclear factor 1 and therefore resemble the α-helical structure associated with NR boxes. Current studies are focused on the function of DRIP150 and the 789–811-amino acid sequence in coactivation of other nuclear receptors including ERα/Sp1- and ERα/API-mediated transactivation and crystallization of the novel α-helical structures in DRIP150 (amino acids 789–811).

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