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AF-2 Activity and Recruitment of Steroid Receptor Coactivator 1 to the Estrogen Receptor Depend on a Lysine Residue Conserved in Nuclear Receptors

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Hormone-dependent transcriptional activation by nuclear receptors depends on the presence of a conserved C-terminal amphipathic α-helix (helix 12) in the ligand-binding domain. Here we show that a lysine residue, which is conserved in most nuclear receptors in the predicted helix 3, is also required for estrogen-dependent transactivation. The replacement of lysine 366 with alanine appreciably reduced activation function 2 (AF-2) activity without affecting steroid- or DNA-binding activity in the mouse estrogen receptor. The mutation dramatically reduced the ability of the receptor to bind steroid receptor coactivator 1 (SRC-1) but had no effect on receptor-interacting protein 140 (RIP-140) binding, indicating that while their sites of interaction overlap, they are not entirely consistent and in keeping with the proposal that the recruitment of coactivators, such as SRC-1, is required for AF-2 activity. Although the function of RIP-140 remains to be established, RIP-140 appears to be capable of recruiting the basal transcription machinery, since overexpression of the protein markedly increased the transcriptional activity of the mutant receptor. Since the lysine residue is conserved, we propose that it is required, together with residues in helix 12, to form the surface by which members of the nuclear receptor family interact with coactivators.

Nuclear receptors are transcription factors whose activity, in many if not all cases, depends on the binding of a hormonal ligand (29, 35). The majority of them consist of three structural domains: an N-terminal domain containing an activation function, AF-1; a DNA-binding domain; and a C-terminal ligand-binding domain containing a second activation function, AF-2 (3, 12). The sequences of the N-terminal activation domains vary considerably in different nuclear receptors, but those for the ligand-binding domain are conserved, suggesting that the mechanism by which this domain functions is likely to be conserved by different family members. Supporting evidence comes from the observation that the regions of the proteins which have been found to be important in receptor dimerization (13, 15, 21, 26, 30) and in ligand-dependent transcriptional activity (2, 10, 11, 38, 49) are similar in different receptors.

Recently the crystal structures for the ligand-binding domains of three nuclear receptors, i.e., RXRα (4), RARγ (37), and TRα (45), revealed that they contain a similar helical fold that results in the formation of a well-conserved hydrophobic ligand-binding pocket. However, one striking difference was the position of the C-terminal helix, helix 12, in RXRα, which was crystallized without the ligand, compared with that in RARγ and TRα, which were crystallized in the presence of their ligands. Since this helix is implicated in AF-2 activity (2, 10, 11, 38), one role for ligand binding appears to be the realignment of helix 12 over the ligand-binding pocket to generate transcriptionally active receptors. Although nuclear receptors bind directly with a number of basal transcription factors in vitro, including the TATA box-binding protein (39), TFIIB (1, 17), and $hTAF_{II}30$ (18), these interactions are unaffected by ligand binding or by mutations in the AF-2 amphipathic α-helix that abolish transcriptional activity. Furthermore, the observation that AF-2 activity can be inhibited by overexpressing the hormone-binding domain in "squelching" experiments (42) suggests that AF-2 is likely to interact with target proteins that are distinct from basal transcription factors. Several candidate target proteins have been identified, namely, RIP-140 and RIP-160 (5, 6), ERAP-140 and ERAP-160 (16), TIF1 (24), a number of isoforms of SRC-1 (20, 33), TRIP1/SUG-1 (25, 44), TIF2 (43), and CBP/p300 (7, 20, 41). To date SRC-1, TIF2, and CBP/p300 have been shown to function as transcriptional coactivators, while the roles of the other proteins are still unclear, although their interaction with mutant receptors correlates with the AF-2 activity of the receptors.

It is doubtful whether helix 12 functions autonomously, at least in the estrogen receptor, since previous experiments suggest that AF-2 is comprised of dispersed elements brought together upon estrogen binding (46). Additional elements could include helix 3 and helix 4, since they are in close proximity to helix 12 in ligand-bound RARα and TRα (37, 45) and since mutation of residues in helix 3 of TRβ1 reduced its transcriptional activity (32). Thus, we compared the sequences of the predicted helices 3 and 4 in nuclear receptors (48) to search for conserved residues that might be required for AF-2 activity. By analyzing the transcriptional activities of mutant receptors, we identified a lysine residue at the predicted C terminus of helix 3 in the estrogen receptor which is required for ligand-dependent transcriptional activity. Since mutation of this residue dramatically reduces the binding of SRC-1 and TIF2, the defect in transcriptional activity may reflect, at least in part, inefficient recruitment of coactivators. The conservation of this lysine residue suggests that it is important for ligand-dependent transcriptional activation by other members of the nuclear receptor family.

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MATERIALS AND METHODS

Abbreviations. The following abbreviations are used in this paper: AF-2, activation function 2; CAT, chloramphenicol acetyltransferase; CBP/p300,

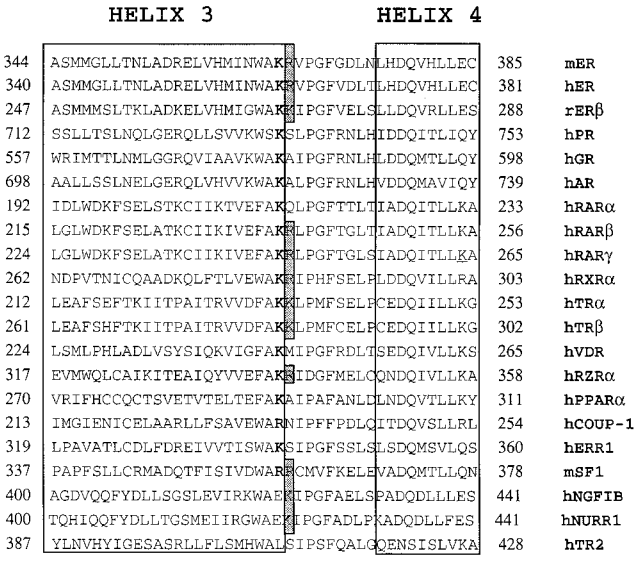

FIG. 1. Alignment of nuclear receptor sequences corresponding to helices 3 and 4 in the ligand-binding domain of human RARγ (hRARγ). Amino acid sequences of members of the nuclear receptor superfamily were aligned based on the conserved fold identified by Wurtz and coworkers (48). Helix 3 and helix 4 are indicated by the large boxes. The well-conserved positively charged lysine or arginine residue at the end of helix 3 is indicated by boldface. The position of the less conserved positively charged residue in the loop between helices 3 and 4 is indicated by shaded boxes. The lysine residue in hRARγ which is implicated in contacting helix 12 is underlined. The following GenBank accession codes were used to retrieve the receptor sequences from database: mouse estrogen receptor (mER), MUSESTRU; human estrogen receptor (hER), HSERR; rat estrogen receptor β (rERβ), RNU57439; human progesterone receptor (hPR), HUMPGRR; human glucocorticoid receptor (hGR), HSGCRAR; human androgen receptor (hAR), HUMANDREC; hRARα, HSRAR; hRARβ, HSHAPRA; hRARγ, HUMRARG; hRXRα, HSRARLP; hTRα, HUMTHRA1A; hTRβ, HSERBAR; human vitamin D receptor (hVDR), HUMVDR; human retinoic Z receptor α (hRZRα), HSU04897; human peroxisome proliferator-activated receptor α (hPPRα), HUMPPAR; human COUP transcription factor 1 (hCOUP1), HSEAR3; human estrogen-receptor related receptor 1 (hERR1), HSSTHOR; mouse steroidogenic factor 1 (mSF1), MUSELP; hNGFIB, HUMTR3A; hNURR1, HSNOT; and human testis receptor 2 (hTR2), HUMTR211.

CREB-binding protein; CSS, charcoal-dextran stripped fetal bovine serum; GST, glutathione *S*-transferase; DMEM, Dulbecco's modified Eagle's medium; ERAP-140; estrogen receptor associated protein 140; ERE, estrogen response element; NGFIB, nerve growth factor-induced receptor B; NURR1, Nur-related receptor 1; RARγ, retinoic acid receptorγ; RXRα, retinoic X receptor α; RIP-140, receptor-interacting protein 140; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SRC-1, steroid receptor coactivator 1; TIF1, transcription intermediary factor 1; and TRα, thyroid hormone receptor α.

Computer analyses. To identify the region of the ligand-binding domain of each nuclear receptor that best corresponds to the third and fourth α-helices predicted for nuclear receptors (48), we used the program BESTFIT of the Genetics Computer Group (University of Wisconsin) package. Alignment of these regions was obtained with the program PILEUP (Genetics Computer Group). The amino acid sequences of nuclear receptors were obtained from the EMBL database.

Plasmids. The following plasmids have been characterized previously: pSP65MOR (10), GAL4-AF2 and GST-AF2 (5), pMT2-mER (22), pBluescript-RIP-140 and pEFBOS-RIP-140 (6), pSG5-TIF1 (24), pSG5-TIF2 (43), pERE-tk-LUC (47), and p$(GAL)_5$CAT (28). The SRC-1 cDNA was amplified from a human B-cell library by using oligonucleotides based on the original sequence (33) and was cloned into pBluescript SK+ and the eukaryotic expression vector pSG5.

In order to create point mutations in the codons coding for lysine 366 and arginine 367 of the mouse estrogen receptor, oligonucleotide cassettes containing the desired nucleotide changes were cloned into pSP65MOR after digestion with *Nde*I and *Xho*I. The oligonucleotides were TATGATCAACTGGGCAGC GAGAGTGCCAGGCTTTGGAGATCTGAATCTCCATGATCAGGTCCAC CTTC and its complementary strand to generate the mutation K366A and TATGATCAACTGGGCAAAGGCGGTGCCAGGCTTTGGAGATCTGAAT CTCCATGATCAGGTCCACCTTC and its complementary strand for the mutation R367A. After verification of the sequences of the mutant receptors, they were transferred, as *Eco*RI fragments, into the mammalian expression vector pMT2 to generate pMT2-K366A and pMT2-R367A. Fusion proteins containing the mutant hormone-binding domains and either the GAL4 DNA-binding domain or GST were generated by transferring *Nde*I/*Sac*I-digested receptor fragments into GAL4-AF2 and GST-AF2 (5).

Transient-transfection experiments. COS-1, HeLa, and ZR75-1 cells were routinely maintained in DMEM containing 10% fetal bovine serum (Gibco) and subcultured once a week. For transient-transfection assays the cells were plated into 24-well microtiter plates in phenol-red-free medium containing 5% CSS. Cells were transfected by using a modified calcium phosphate coprecipitation method (9) with 1 μg of reporter plasmid, 200 ng of pCMV-βGal plasmid as an internal control, various amounts of expression plasmids as detailed in the figure legends, and pBluescript plasmid (Stratagene), if necessary, to a total of 1.5 μg of DNA per well. The reporters were either pERE-tk-LUC (47), containing the vitellogenin A2 ERE upstream of the thymidine kinase promoter in the plasmid pGL2 (Promega), or p$(GAL)_5$CAT (28), containing five copies of the GAL4 DNA-binding site upstream of the E1b TATA box. In some experiments pEFRIP (6) or pSG5-SRC-1 was added, as indicated in the figure legends. After 24 h, the cells were washed with DMEM and treated with medium containing 5% CSS and either the ethanol vehicle, 10^{-8} M 17β-estradiol, 10^{-7} M 4-hydroxytamoxifen, or 10^{-7} M ICI 182,780 for 24 h. The cells were then washed with phosphate-buffered saline and harvested in lysis buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 150 mM NaCl, and 0.65% Nonidet P-40). Extracts were assayed for luciferase activity (10), CAT activity (40), or β-galactosidase activity, as a control, using a Galacto-Light chemiluminescence assay (Tropix).

For biochemical analysis, the wild-type and mutant receptors were overexpressed in COS-1 cells. After electroporation of the cells at 450 V and 250 μF in the presence of 20 μg of plasmid DNA, they were plated out in DMEM containing 5% CSS and grown for 48 h. Whole-cell extracts were prepared in buffer containing 0.4 M KCl, 20 mM HEPES (pH 7.4), 1 mM dithiothreitol, and 20% glycerol. The protein contents of cell extracts were determined by a colorimetric method (Bio-Rad).

Western blotting. Aliquots of whole-cell extracts containing equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes by electroblotting overnight. The membranes were blocked in TBS-T (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, 0.1% Tween 20) containing 5% nonfat milk powder, washed with TBS-T, and incubated for 1 h with monoclonal antibody H222 raised against the human estrogen receptor. After being washed, the membranes were incubated with rabbit anti-rat immunoglobulins (DAKO) and washed again with TBS-T. The bound immunoglobulins were visualized by using the ECL detection system (Amersham Life Science).

Gel retardation and ligand-binding assays. To determine the DNA-binding activities of the mutant receptors, aliquots of whole-cell extracts that contained 0.5 μg of protein were incubated with ^{32}P-labelled and annealed oligonucleotides corresponding to a 32-bp fragment of the vitellogenin A2 gene promoter containing a consensus ERE. Binding reactions were performed in the presence of ethanol or 10^{-6} M 17β-estradiol, and the antibody MP16, specific to estrogen receptors (14), was added to some reaction mixes, as indicated in the legend to Fig. 3. Receptor-DNA complexes were separated from unbound DNA in non-denaturing polyacrylamide gels and visualized by autoradiography.

Ligand-binding analyses of the wild-type and mutant receptors were performed as described previously (13), with [^{3}H]estradiol (Amersham International). Scatchard analysis was performed over the range of 0.3 to 30 nM labelled steroid in the absence or presence of a 100-fold excess of unlabelled estradiol.

GST pull-down assays. The ligand-binding domains of wild-type and mutant estrogen receptors were expressed as GST fusion proteins in *Escherichia coli* as described previously (5). The expression of correctly sized proteins was monitored by SDS-PAGE. For GST pull-down assays, bacterially expressed GST or GST fusion proteins were bound to glutathione-Sepharose 4B beads (Pharmacia Biotech). RIP-140 and SRC-1 cDNAs in pBluescript vectors (Stratagene) and TIF1 and TIF2 in pSG5 were used to generate [^{35}S]methionine (Amersham International)-labelled proteins by using the TNT coupled in vitro translation system (Promega). These ^{35}S-labelled proteins were incubated with beads containing either GST or GST fusion proteins in the absence or presence of 10^{-6} M estradiol or 4-hydroxytamoxifen in NETN buffer (0.5% Nonidet P-40, 20 mM Tris-HCl [pH 8.0], 200 mM NaCl, 1 mM EDTA) containing protease inhibitors. After an overnight incubation, free proteins were washed away from the beads with NETN buffer. Bound proteins were extracted into loading buffer, separated by SDS-PAGE, and visualized by fluorography. Before fluorography, the gels were stained with Coomassie blue to ascertain that equal amounts of GST fusion protein were bound to the beads in all samples. The amounts of bound ^{35}S-labelled proteins were quantified with a PhosphorImager (Molecular Dynamics).

RESULTS

Identification of a conserved lysine in helix 3 of the ligand-binding domains of nuclear receptors. The crystal structures of the ligand-binding domains of RARγ (37) and TRα (45) bound to their cognate ligands indicate that helix 12, which is

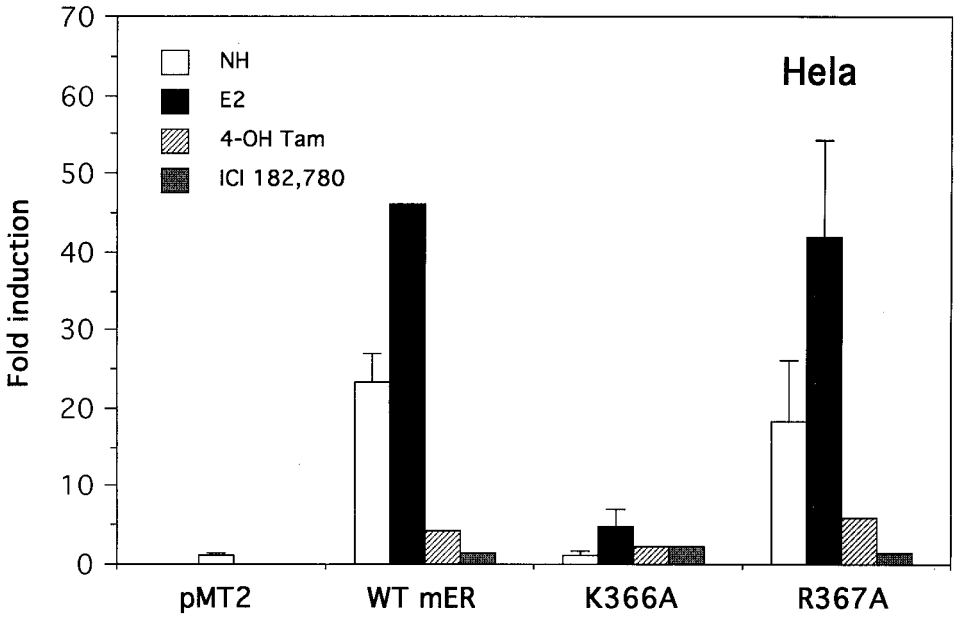

FIG. 2. Analysis of the transcriptional activities of mutant receptors in HeLa cells. HeLa cells were transiently transfected with 50 ng of the wild-type receptor (WT mER), the mutant receptor K366A or R367A, or the parental vector (pMT2). After transfection, cells were treated with medium containing no added hormone (NH), 10^{-8} M 17β-estradiol (E2), 10^{-7} M 4-hydroxytamoxifen (4-OH Tam), or 10^{-7} M ICI 182,780 for 24 h. Luciferase activity was normalized with β-galactosidase activity, and data is expressed as fold induction over activity of the vector control. The data for NH and E2 was derived from four separate experiments containing duplicate samples, and means ± standard errors are given.

required for AF-2 activity, is in close proximity to helix 3 or helix 4. Since helix 12 was found to be stabilized by the formation of a salt bridge formed between Glu-414 and Lys-264 in RARγ (37), we compared the sequences of helices 3 and 4 in nuclear receptors (48), focusing on basic amino acids to search for residues that might serve a similar role. Although a lysine corresponding to that in helix 4 in RARγ was not present in steroid hormone receptors, we identified a lysine residue at the C terminus of helix 3 that was conserved in all ligand-dependent nuclear receptors and many orphan receptors, as shown in Fig. 1. The only vertebrate receptors lacking the conserved lysine were TR2, where there is a leucine (8), and NGFIB/NURR1 family members, where there is a glutamic acid (23, 31). Adjacent to this lysine, a positively charged residue was also present in many but not all receptors. We therefore investigated the importance of Lys-366 and Arg-367 in transcriptional activation by the estrogen receptor by replacing them with an alanine residue to generate K366A and R367A, respectively.

Transcriptional activation by mutant mouse estrogen receptors. We compared K366A and R367A with the wild-type receptor for the ability to stimulate transcription from an ERE-thymidine kinase-luciferase reporter gene in transiently transfected HeLa cells. The wild-type receptor stimulated transcription approximately 20-fold over that of controls, and this was increased a further 2-fold by the addition of 17β-estradiol (Fig. 2). As previously discussed (10), the transcriptional activity observed in the absence of added 17β-estradiol is probably due to the presence of residual estrogen in the stripped serum, since it was reduced to fourfold in the presence of 4-hydroxytamoxifen and was negligible after the addition of the pure antiestrogen ICI 182,780. The activity of R367A was similar to that of the wild-type receptor, whereas that of K366A was markedly reduced in the presence of 17β-estradiol and negligible in the presence of the antiestrogens. Analogous results were obtained with a range of different amounts of expression plasmids. Thus, we conclude that Lys-366, but not Arg-367, is required for transcriptional activity of the estrogen receptor.

Mutation of Lys-366 does not affect steroid or DNA binding. To establish whether Lys-366 was directly involved in transcriptional activation, we investigated whether the mutation affected the expression of the receptor or its ability to bind estradiol or DNA. The wild-type and mutant receptors were expressed in COS-1 cells, and initially their relative amounts in cell extracts were determined by Western blotting analysis with the H222 monoclonal antibody specific for the estrogen receptor. Figure 3A shows that K366A, R367A, and the wild-type receptor were all expressed at similar levels, with variations insufficient to account for the alterations in transcriptional activity. We then analyzed their estrogen-binding activities by Scatchard analysis with ^{3}H-17β-estradiol. The K_d values (means ± standard errors) were 0.53 ± 0.44 nM for the wild-type receptor, 0.74 ± 0.20 nM for K366A, and 1.16 ± 0.46 nM for R367A, indicating that the two mutations do not appreciably reduce the affinity of the domain for estradiol. Thus, all of the receptors would be expected to be saturated with the hormone at the concentration used in the transfection experiments, namely, 10^{-8} M. We next analyzed the DNA-binding properties of the mutant receptors by using the vitellogenin A2 consensus ERE sequence in gel retardation assays. Both mutant receptors retained their ability to bind to DNA (Fig. 3B), and their mobilities were retarded by the presence of the estrogen receptor antibody MP16, as has been previously found for the wild-type receptor (14). Given that the mutations do not significantly affect these properties of the receptor, we conclude that the overall structure of the mutant receptors resembles that of the wild-type receptor.

Lys-366 is required for AF-2 activity. To test whether the disruptive effect of the lysine mutation on transcriptional ac-

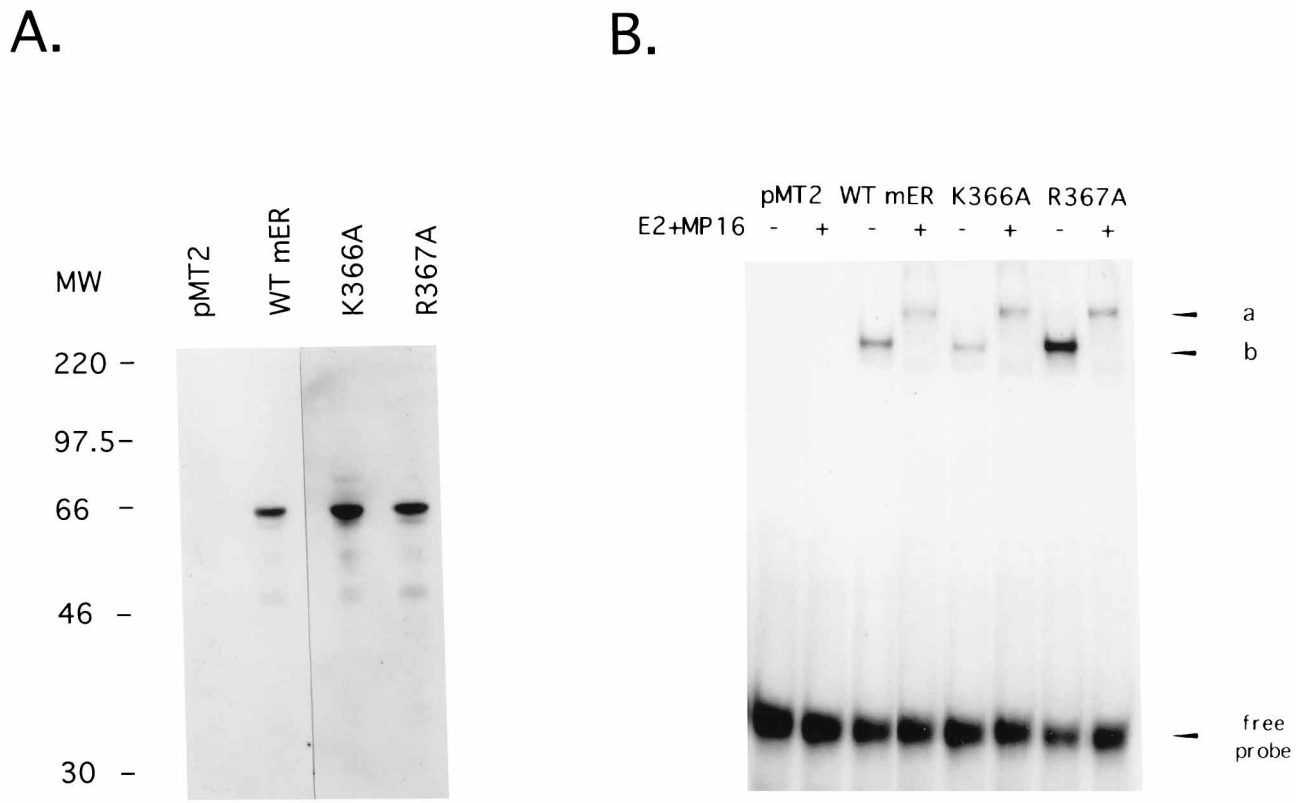

FIG. 3. Expression and DNA-binding activities of wild-type and mutant estrogen receptors. The wild-type (WT mER) and K366A or R367A mutant receptors were transiently expressed in COS-1 cells after electroporation with pMT2-based expression vectors or the parent vector (pMT2) as a control. Cell extracts were prepared as detailed in Materials and Methods. (A) Analysis of expression by Western blotting with monoclonal antibody H222 raised against the human estrogen receptor. The positions of molecular weight (MW) markers (in thousands) are shown on the left. (B) Analysis of DNA-binding activity by gel shift analysis. Estrogen receptor-DNA complexes were formed in the absence or presence of estradiol (E2) and MP-16 antiserum as indicated. Arrows indicate the positions of complexes observed in the absence (arrow a) or presence (arrow b) of MP16 antiserum.

tivity was restricted to AF-2 or depended on the presence of AF-1, we fused the wild-type and mutated ligand-binding domains to the DNA-binding domain of GAL4 to generate GAL4-AF2, GAL4-K366A, and GAL4-R367A. The ability of GAL4-AF2 to stimulate transcription of a GAL4 reporter in HeLa cells was dependent on the presence of 17β-estradiol (Fig. 4A). In contrast, GAL4-K366A had extremely low activity, in accordance with the results obtained with full-length receptors (Fig. 2). However, the arginine mutation, which had little effect on the activity of the full-length receptor in HeLa cells (Fig. 2), reduced the activity of GAL4-R367A to 40% of that of GAL4-AF2 (Fig. 4A). Similar experiments were performed with COS-1 cells and ZR75-1 human breast cancer cells, in which we observed that the activity of GAL4-K366A was also negligible while that of GAL4-R367A was 50 to 60% that of GAL4-AF2 (Fig. 4A). Thus, in the absence of AF-1, it appears that the arginine mutation slightly reduced AF-2 activity, but Western blot analysis indicated this was partly accounted for by a reduction in the expression of GAL4-R367A (Fig. 4B). On the other hand, the amount of GAL4-K366A was approximately four times that of the wild-type receptor, and so we conclude that Lys-366 is crucial for AF-2 activity in the estrogen receptor.

Lys-366 is important for the binding of SRC-1, TIF1, and TIF2 but not RIP-140. Previous work has shown that the ligand-binding domains of nuclear receptors are able to bind a number of putative coactivators (6, 20, 24, 25, 33, 43, 44) and that the interaction with mutant receptors correlates with their transcriptional activity. We therefore tested the effect of the lysine and arginine mutations on the ability of the estrogen-binding domain, fused to GST, to interact with RIP-140 and SRC-1. As previously observed (6, 19a), RIP-140 and SRC-1, translated in vitro, were retained by GST-AF2 but not GST alone, and the interaction was enhanced by estradiol but not 4-hydroxytamoxifen (Fig. 5). In contrast, while GST-K366A retained estrogen-dependent binding of RIP-140, that of SRC-1 was dramatically reduced to 3% of that of GST-AF2. As expected, the ability of GST-R367A to retain RIP-140 and SRC-1 was unaffected by the mutation. Similar experiments with TIF1 and TIF2 showed that their binding properties resembled those of SRC-1, as they did not bind efficiently to GST-K366A (Fig. 5B and C). Thus, we conclude that the binding site of RIP-140 is likely to overlap that of SRC-1, TIF1, and TIF2 but is not entirely coincident since these proteins require Lys-366 for their interaction.

RIP-140 can partially restore the activity of K366A. In view of the differences in the abilities of SRC-1 and RIP-140 to interact with K366A in vitro, we analyzed their effects on the activity of the mutant receptor in transfected HeLa cells. The estrogen-stimulated activity of the wild-type receptor was enhanced up to fivefold with increasing amounts of SRC-1 (Fig. 6A) and up to twofold with RIP-140, although this was abrogated as the amount of RIP-140 was increased (Fig. 6B). Although K366A was a poor transcriptional activator, as described above, its activity was slightly restored at the highest concentration of SRC-1 (Fig. 6A). Surprisingly, RIP-140 also

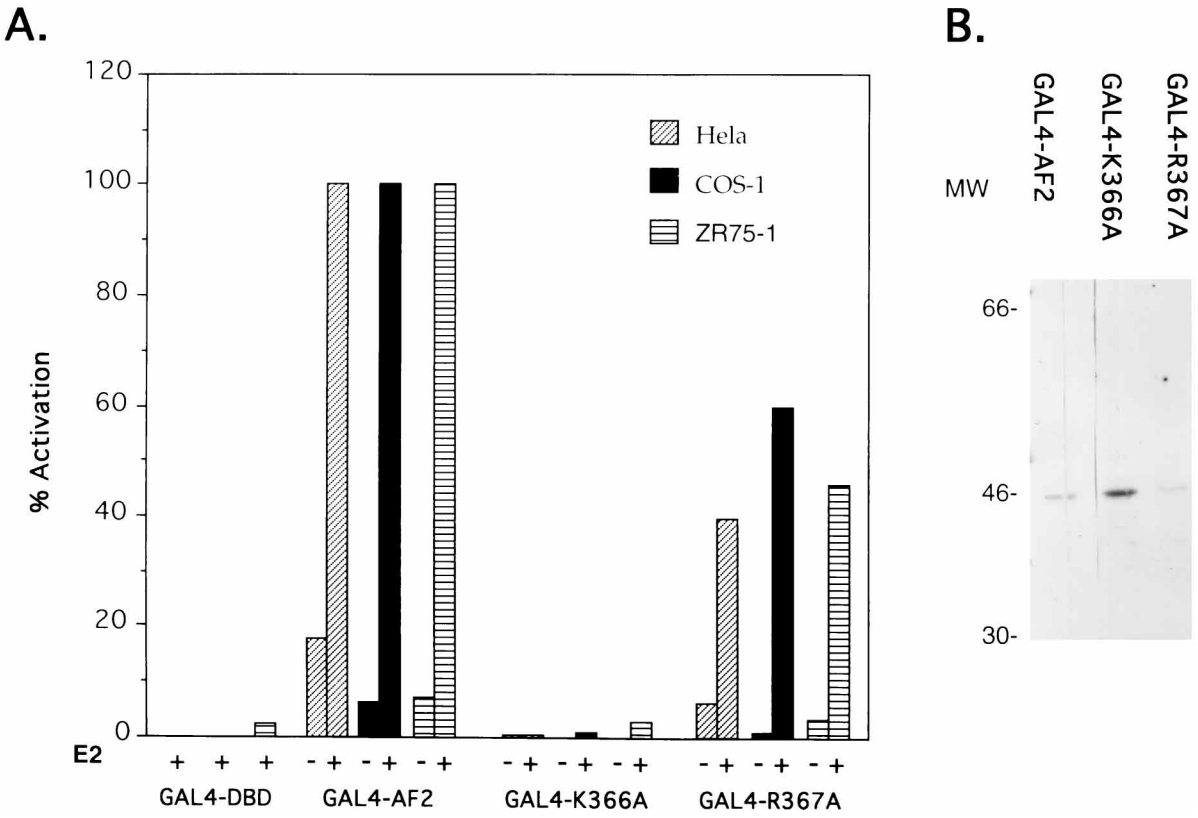

FIG. 4. Mutation of Lys-366 abolishes AF-2 activity in the mouse estrogen receptor. (A) HeLa, COS-1, and ZR75-1 cells were transfected with GAL4 DNA-binding domain, GAL4-AF2, GAL4-K366A, or GAL-R367A with calcium phosphate coprecipitation with 150 ng of GAL4 plasmid for HeLa cells and 50 ng for COS-1 and ZR75-1 cells. After transfection, cells were treated with medium containing no added hormones (−) or 10^{-8} M 17β-estradiol (+) for 24 h. The CAT activities were normalized with β-galactosidase activity to correct for differences in transfection efficiencies. The data is expressed as the percentage of the normalized CAT activity of GAL4-AF2 in the presence of 17β-estradiol in each cell line. (B) Western blot analysis of GAL4-AF2, GAL4-K366A, and GAL-R367A transiently expressed in COS-1 cells. The blot was developed with monoclonal antibody H222 specific for the estrogen receptor. The positions of molecular weight (MW) markers (in thousands) are shown on the left.

stimulated the activity of K366A up to 40-fold at the optimum concentration, but this too was reduced with increasing amounts of RIP-140. Thus, the effect of RIP-140 on K366A was much more pronounced than that on the wild-type receptor. The effects of SRC-1 and RIP-140 on the transcriptional activity of R367A were similar to those on the wild-type receptor (data not shown), as expected, since their in vitro binding activities were similar (Fig. 5). Thus, we conclude that while the endogenous levels of RIP-140 are not sufficient to maintain the transcriptional activity of K366A, RIP-140 is able to stimulate the activity of this mutant receptor when overexpressed.

DISCUSSION

The ligand-binding domain of nuclear receptors is a multifunctional domain which forms the ligand-binding pocket, provides a dimerization surface, and harbors the ligand-dependent transcriptional activation function AF-2 (29, 35). Recently, elucidation of the crystal structure of this domain in three nuclear receptors revealed that they all share a common fold comprising 11 or 12 helices and a β-sheet folded into an antiparallel α-helical sandwich (4, 37, 45). Furthermore, alignment of other nuclear receptor sequences predicted that this fold is likely to be universal in all members of the superfamily (48). In this paper, we have applied these structural predictions to the mouse estrogen receptor. First, we show that by aligning nuclear receptor sequences we were able to identify a well-conserved lysine residue at the predicted C terminus of helix 3 (48). The replacement of this lysine in the mouse estrogen receptor with alanine (K366A) dramatically reduced estrogen-dependent transcriptional activity without affecting steroid- or DNA-binding activities. Analysis of the transcriptional activities of chimeric proteins in which the ligand-binding domain was fused to the GAL4 DNA-binding domain demonstrated that this lysine was crucial for AF-2 activity. In contrast, the mutation of an adjacent arginine residue, which is also conserved in many receptors, only slightly reduced estrogen-dependent transcriptional activity and is likely to have a distinct function.

Nuclear receptor signature region and helix 12. The conserved lysine residue maps to a region whose sequence is extremely well conserved within the nuclear receptor superfamily. This region of 34 amino acids, which has been called the nuclear receptor signature region (48), encompasses the C-terminal half of helix 3, all of helix 4, and the loop between them. Although a specific function has not been assigned to this part of nuclear receptors, a number of studies implicate the signature region in transcriptional activation. First, the transcriptional activity of the rat TRβ1 was reduced when the conserved lysine and the preceding alanine were replaced with isoleucine and aspartic acid, respectively (32). In addition, the transcriptional activity of the human estrogen receptor was reduced about threefold when three mutations were intro-

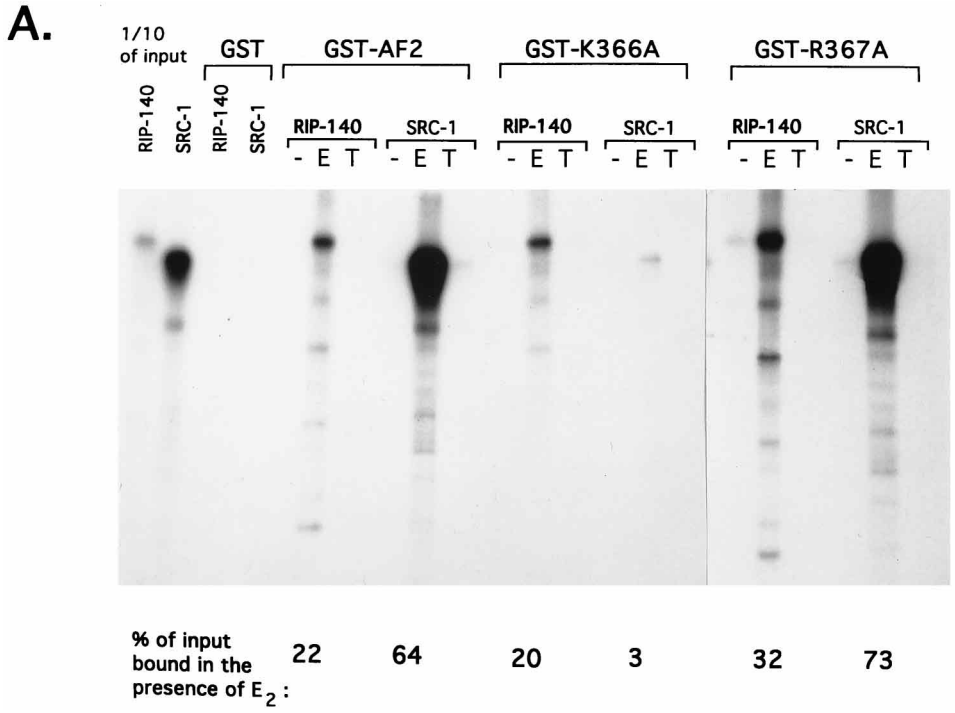

FIG. 5. Mutation of Lys-366 disrupts the 17β-estradiol-dependent interaction between the receptor and the coactivator SRC-1. GST alone and the GST fusion proteins GST-mER, GST-K266A, and GST-R367A were expressed in *E. coli* and immobilized on glutathione-Sepharose beads. In vitro-translated RIP-140 or SRC-1 (A), TIF2 (B), and TIF1 (C) were incubated with the beads in the absence of added hormone (−) or in the presence of 10^{-6} M 17β-estradiol (E) or 10^{-6} M 4-hydroxytamoxifen (T), as indicated. The bound proteins were analyzed by SDS–10% PAGE and visualized by fluorography. The bottom of panel A shows results of quantifications performed with a PhosphorImager. As a control, 1/10 of the amount of ^{35}S-protein included in the binding reactions is shown in the first lane(s) of each panel.

duced into helices 4 and 5 (34). Finally, mutations in the signature region of the human androgen receptor have been identified in patients with partial or complete androgen insensitivity syndrome (36). Therefore, these observations, together with our results demonstrating that lysine 366 is crucial for the activity of the estrogen receptor, suggest that the conserved signature region may be required for the formation of AF-2.

Previous work has established the importance of helix 12 for AF-2 activity. This amphipathic α-helix comprises two well-conserved pairs of hydrophobic residues pointing towards the core of the ligand-binding domain and negatively charged residues exposed on its surface (37, 45). Upon ligand binding, this helix seems to be realigned to bring it into close proximity with helix 3 and/or helix 4 (4, 37, 45). That helix 12 does not function autonomously is suggested not only by the observation that the conserved lysine in helix 3 is necessary for AF-2 activity but also by the observation that mutation of residue K264 in helix 4 of human RARγ also almost abolished transactivation (37). This residue was shown to form a salt bridge with glutamic acid residues in helix 12 (E414 and to a lesser extent E417), thus stabilizing the active structure (37). This salt bridge may not be a general feature of all nuclear receptors, since the corresponding lysine residue is not conserved in steroid hormone receptors (Fig. 1) and since there was no evidence for a salt bridge between the negatively charged residues in helix 12 and any of the lysines in helix 3 or in helix 4 in the TRα structure (45). Since both the charged residues in helix 12 and residues in the signature region, including the lysine residue in helix 3, are exposed on the surface of the ligand-binding domain (37, 45), it is possible that they generate a hydrophilic surface which interacts with downstream target proteins required to mediate AF-2 activity.

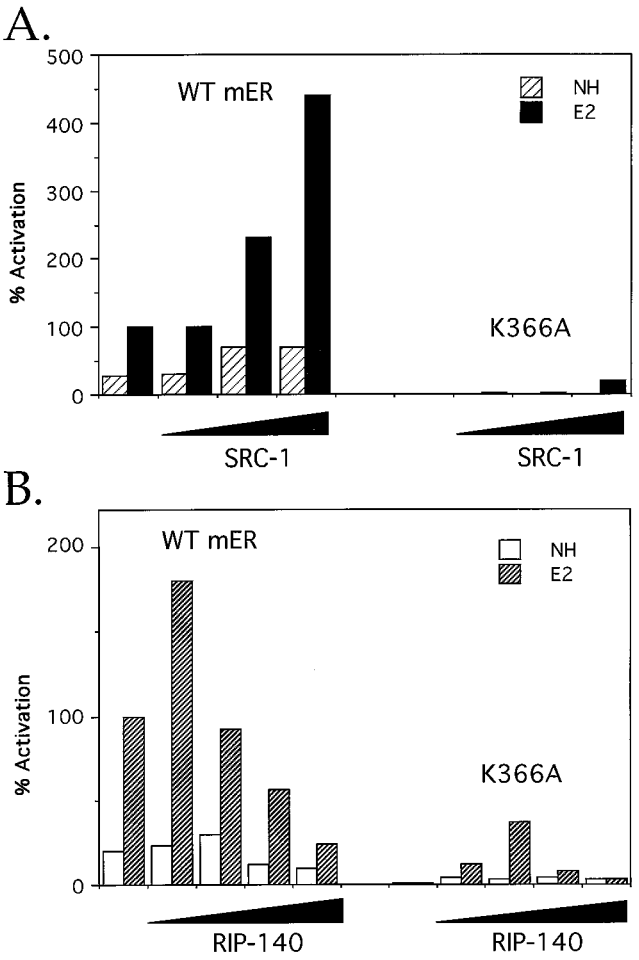

FIG. 6. Ability of RIP-140 and SRC-1 to modulate the transcriptional activity of K366A. HeLa cells were transiently transfected with the wild-type (WT mER) or K366A mutant receptor (50 ng of expression vector) in the absence or presence of increasing amounts of SRC-1 (50, 100, or 300 ng) (A) or RIP-140 (0.1, 0.5, 5, or 300 ng) (B) expression vector. After transfection, the cells were treated with medium containing no added hormone (NH) or 10^{-8} M 17β-estradiol (E2) for 24 h. The data is expressed as the percentage of the normalized luciferase activity observed in the absence of exogenously expressed SRC-1 or RIP-140.

Although the lysine residue in helix 3 and the C-terminal amphipathic α-helix are conserved in all ligand-dependent nuclear receptors and the vast majority of orphan receptors (Fig. 1), they appear to be absent in TR2 and NGFIB/NURR1 family members. It is unclear whether these proteins contain an AF-2 activity, and it is conceivable that they may be activated not by ligand binding but by, for example, phosphorylation. They are transiently expressed in response to mitogenic signals (23, 31) and, like the products of other immediate-early genes, may be targets for specific kinase cascades.

AF-2 as a target site for coactivators. It is likely that the function of AF-2 is modulated by target proteins whose concentrations are limiting in cells (42). Many potential candidates that interact with the ligand-binding domain have been identified, including RIP-140 and RIP-160 (5, 6), ERAP-140 and ERAP-160 (16), TIF1 (24), a number of isoforms of SRC-1 (20, 33), TRIP1/SUG-1 (25, 44), TIF2 (43), and CBP/p300 (7, 20, 41). Their interaction is enhanced in the presence of the ligand, and they fail to interact with mutant receptors containing inactivating mutations in helix 12 (6, 19a, 24, 25, 43, 44). In this paper we show that the binding of RIP-140 to the estrogen receptor can be distinguished from that of a number of other coactivators, including SRC-1, TIF1, and TIF2. The transcriptionally inactive mutant receptor K366A was found to be defective for binding to the latter proteins but not to RIP-140. This is consistent with the observation that SRC-1 and TIF2 function as coactivators for the estrogen receptor (33, 43) and suggests that the transcriptional defect of the mutant receptor results from its inability to recruit coactivators upon ligand binding. Although the normal cellular concentrations of SRC-1 may not be sufficient to interact with K366A, its transcriptional activity was slightly increased when SRC-1 was overexpressed (Fig. 6). The limited ability of SRC-1 to rescue the activity of K366A may reflect its very weak interaction or the inability of the receptor to interact with other coactivator proteins, such as CBP/p300 (7, 20, 41), required for optimum AF-2 activity.

Previous work has established that the binding of both RIP-140 and SRC-1, TIF1, and TIF2 (6, 19a, 24, 43) is dependent on the integrity of helix 12, suggesting that they contact overlapping sites on the receptor. Our results with the lysine mutation, which discriminates between RIP-140 and the other cofactors, indicates that their sites of interaction cannot be coincident. Moreover, the transcriptional defect in K366A does not seem to be related to the lack of RIP-140 binding, but nevertheless, its transcriptional activity was appreciably stimulated when RIP-140 was overexpressed. Analogous results in which RIP-140 was found to increase the transcriptional activity of the estrogen receptor were obtained for yeast (19). The function of RIP-140 remains unclear, but given that it can stimulate the transcription of reporter genes in transfected cells when it is fused to a DNA-binding domain (27), we propose that it acts as a bridging protein between the ligand-binding domain of some nuclear receptors and the basal transcription machinery.

It is currently difficult to assess which of the many proteins that have been shown to interact with the ligand-binding domain of nuclear receptors are actually recruited in intact cells, because very little is known about their relative levels or affinities. The mutation of the conserved lysine in this study not only established that it was crucial for AF-2 activity but also supported the view that recruitment of SRC-1 or TIF2, or other coactivators, is essential, and finally, it provided information about the binding sites of coactivators. The identification of additional mutations that discriminate between different receptor-interacting proteins is likely to be very useful in analyzing their different functions.

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