THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 12, Issue of March 20, pp. 6679–6688, 1998
Printed in U.S.A.

Enhancement of Estrogen Receptor Transcriptional Activity by the Coactivator GRIP-1 Highlights the Role of Activation Function 2 in Determining Estrogen Receptor Pharmacology*

(Received for publication, September 10, 1997, and in revised form, November 21, 1997)

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The human estrogen receptor (ER) contains two major activation functions (AFs) responsible for its transcriptional activity. One of these, activation function 2 (AF-2), located within the hormone-binding domain (HBD), has been shown to mediate the ligand-dependent transcriptional activity of ER as well as other members of the nuclear receptor superfamily. Recently, proteins interacting with the HBD of several nuclear receptors have been cloned. One of these proteins, glucocorticoid receptor interacting protein (GRIP-1), has been shown to interact with ER and was originally hypothesized to mediate its transcriptional activity through AF-2. However, we find in this study that the transcriptional activity of ER, containing mutations in the AF-2 core sequence, can be enhanced by coexpression of the coactivator GRIP-1, suggesting that this protein may not rely solely on the AF-2 domain for interaction. We propose, therefore, that the HBD of ER either contains multiple binding sites that are necessary for association with GRIP-1 or, alternatively, that this coactivator contacts the receptor in an undetermined region within the HBD. Importantly, these studies demonstrate also that mutations or deletion of AF-2 alter the ligand pharmacology of the receptor such that ER loses the ability to discriminate between agonists and antagonists. Interestingly, on these mutant receptors GRIP-1 still functions as a coactivator independent of the nature of the bound ligand. It is likely, therefore, that the C-terminal AF-2 domain may function as a molecular switch allowing the wild-type receptor to discriminate between agonists and antagonists as well as providing a surface with which associated proteins can interact.

The human estrogen receptor (ER)[1] belongs to a superfamily of ligand-activated nuclear transcription factors (1). Among its members are the receptors for steroid hormones, retinoic acid, vitamin D, thyroid hormone (T3), and a group of orphan receptors for which ligands have yet to be identified (2, 3). The classical models of the ER signal transduction pathway involves binding of the steroidal ligand estradiol to the receptor followed by a conformational change in receptor structure (4, 5). This event facilitates the dissociation of the receptor from an inhibitory multi-protein complex composed of HSP90, HSP70, p59, and possibly other factors (6). Once released from the inhibitory complex, the receptor forms homodimers (7) and subsequently binds DNA elements called estrogen response elements (EREs) located within the regulatory regions of target genes (8, 9). The precise mechanism by which the DNA-bound receptor alters gene transcription has yet to be elucidated. In addition to this well characterized pathway, ER has also been shown to modulate transcription in an ERE-independent manner presumably through its association with other DNA bound factors (10–13). However, the physiological significance of this alternate pathway remains to be determined.

Although the mechanism by which ER regulates gene transcription is unknown, the domains responsible for its transcriptional activity have been well characterized. The major activation functions, AF-1 and AF-2, are located within the N and C termini, respectively (14, 15). A third activation function, AF2a, located within the receptor HBD has also recently been identified (16, 17). It has been shown that in some cell contexts that AF-1 and AF-2 function independently of each other, whereas in others, full transcriptional activity requires both activation domains (15, 18, 19). Functionally, these AFs differ in that AF-2 is located within the ligand-binding domain of ER and requires ligand to manifest its activity. ER antagonists are thought to function by inhibiting the activity of AF-2. Unlike AF-2, AF-1 is a constitutive activator, and in contexts where AF-1 alone is required for transcriptional activity, ER antagonists like tamoxifen manifest partial agonist activity (14, 18). Thus, it appears that cellular factors that interact with either AF-1 or AF-2 are important determinants of the pharmacology of ER ligands.

Lately, considerable progress has been made in understanding how ligands influence the activity of the AF-2 domain within the nuclear receptors. Of particular importance in this regard was the resolution of the crystal structures of RXRα (20), RARγ (21), TRβ (22), and ER (23). These studies reveal that the ligand-binding domain of the nuclear receptors share a common structural motif which is composed of 11 to 12 individual α helices with helix 2 being absent in some members of the superfamily (24). Of particular interest was the observation that the most carboxyl region, helix 12, containing the sequence thought to be responsible for AF-2 activity, was shown to realign over the ligand-binding pocket when associated with agonists. This observation confirmed previous bio-

* This work was supported by National Institutes of Health Research Grant DK 48807 (to D. P. M.) and by California Breast Cancer Research Program of the University of California Grant 2RB-0174 (to M. R. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ER, estrogen receptor; ERE, estrogen response elements; HBD, hormone-binding domain; AF, activation function; GRIP-1, glucocorticoid receptor interacting protein; GST, glutathione *S*-transferase; wt, wild type; CMV, cytomegalovirus; CBP, CREB-binding protein.

chemical studies that predicted major structural alterations within this region upon binding hormone (4, 25, 26). Taken together, these studies suggest that these agonist-induced alterations in ER structure permit the formation of a surface with which ER interacts with the general transcription machinery.

To date, several factors interacting with the HBD of the nuclear receptors have been identified. Among these are several isotypes of SRC-1 (27, 28), GRIP-1 (29), TIF-2 (30), mSUG1 (31), ERAP 140 and ERAP 160 (32), TIF-1 (33), RIP140 and RIP160 (34, 35), and several others (36, 37). Interestingly, *in vitro* interaction studies determined that a region containing helix 12 appeared to be required for interaction with several of these proteins (30, 31, 38). The link between helix 12, AF-2, and the ability of coactivators to bind the nuclear receptors was established when it was demonstrated that mutations which were known to abolish AF-2 transcriptional activity were also shown to abolish the interaction of the nuclear receptors with several of their associated proteins (30, 31, 38–41). This link appeared also to explain how antagonists operate. Since it was determined that antagonists differentially affect HBD structure, it was proposed that the resultant conformation was unable to engage the cofactors required for transcriptional activity. The inability of the coactivator proteins to engage the nuclear receptors in the presence of antagonists supports this hypothesis (27, 31, 38). In this study, we analyzed the relative contribution of the different transcriptional domains of ER in mediating the activities of the coactivator GRIP-1. We show in yeast and in mammalian cells that activation of the estrogen receptor by GRIP-1 can occur in the absence of a functional AF-2. This would suggest that GRIP-1 interacts with the receptor in an unidentified region of the HBD and that perhaps this domain contains several surfaces with which receptor-associated proteins can interact. We also find that unlike the wild-type receptor, which upon binding ER antagonists fails to engage GRIP-1, the transcriptional activity of the AF-2 mutant receptors in the presence of several different classes of ER ligands is enhanced by overexpression of this coactivator. This suggests that it is the contribution of the AF-2 region to receptor structure, rather than its ability to manifest independent transcriptional activity, that is important in determining the agonist/antagonist character of ER ligands.

MATERIALS AND METHODS

Enzymes and Chemicals—Restriction and modification enzymes were obtained from Promega (Madison, WI), Boehringer Mannheim, or New England Biolabs Inc. (Beverly, MA). 17β-Estradiol and 4-hydroxytamoxifen were purchased from Sigma. Oxalyticase was purchased from Enzogenetics (Corvallis, OR). Polymerase chain reaction reagents were purchased from Perkin-Elmer or Promega. ICI182,780 was a gift from Dr. Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK).

Plasmids—Plasmids expressing ER mutants (pER-LL, pER-ML, and pER-535-stop) were generated using oligo-directed mutagenesis as described previously (42). pGAD424 was purchased from CLONTECH. Yeast ER expression vector YEP3X was given as a gift by Delores Santiso-Mere (Ligand Pharmaceuticals, San Diego, CA). YEPE28 (ER179C) has been described previously (19). The yeast estrogen-responsive β-galactosidase reporter construct (YRPE2) has been described elsewhere (19). pCMV.HA/GRIP1, coding for GRIP1 amino acids 322–1121, was reported elsewhere (38). The estrogen-responsive mammalian reporter plasmids C3-Luc and 3x-ERE-TATA-Luc have been described previously (18, 42). Mammalian expression vectors for ER and ER mutants (ER-wt, ER179C, ER-TAF1, and ER-Null) were also described previously (18). All products of polymerase chain reaction-based cloning were sequenced to ensure the fidelity of the resultant construct.

β-Galactosidase Assays in Yeast—The transcriptional activities of ER, and ER mutants, were assayed on the ERE-CYC1-β-galactosidase reporter gene as described previously (43). Briefly, yeast strain YPH500 (Mat a *ura*3-52, *lys*2-801, *ade*2-101, *trp*1Δ63, *his*3Δ200 *leu*2Δ1) was transformed with pGRIP1/fl (38) or the empty expression vector pGAD424 along with the wtER or ER mutant expression plasmids. Resultant transformants were grown to OD 0.8 and incubated with media supplemented with copper and increasing concentrations of estradiol. After a 4-h incubation with hormone, the yeast cell wall was lysed using a buffer consisting of 0.1% SDS and the enzyme oxylyticase. Extracts were then analyzed for β-galactosidase activity using the substrate *o*-nitrophenyl-β-D-pyranoside.

Cell Culture and Transient Transfection Assays—HepG2 and HeLa cells were maintained in modified Eagle's medium (MEM) (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.). Cells were plated in 24-well plates (coated with gelatin for transfections of HepG2 cells) 24 h prior to transfection. DNA was introduced into the cells using Lipofectin (Life Technologies, Inc.) as described previously (44). Typically, 1500 ng of reporter, 1000 ng of receptor, 500 ng of coactivator, and 20 ng of normalization vector were used for each transfection performed in triplicate. Cells were incubated with the DNA/Lipofectin mixture either 2 or 3 h for transfections of HeLa and HepG2 cells, respectively. Cells were washed with phosphate-buffered saline (PBS) and incubated with the indicated hormone diluted in phenol red-free modified Eagle's medium (Life Technologies, Inc.) supplemented with charcoal-stripped fetal bovine serum (HyClone, Logan, UT) 24 h prior to harvesting. pCMV-βgal (Stratagene, La Jolla, CA), containing the bacterial *lacZ* gene expressed from the SV40 early promoter, was also introduced into the cells and used as an internal control to account for transfection efficiency. After harvesting, cells were assayed for β-galactosidase and luciferase activity as described previously (44).

Interaction of GRIP-1 with ER and ER Mutants—Glutathione *S*-transferase (GST) pull-downs with GST-GRIP1 fusion protein and ER proteins were performed essentially as described (29). [^{35}S]Methionine-labeled ER or ER mutant were synthesized using *in vitro* transcription and translation from the cognate mammalian expression vectors described above. The resultant labeled proteins were incubated with Sepharose beads containing GST-GRIP-1 and eluted by boiling in SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis.

Western Immunoblot Analysis—Western blots were prepared from nuclear extracts isolated from HeLa cells transfected with wtER alone or in combination with GRIP-1. Nuclear extracts were prepared as described previously (45). 15 μg of total protein was denatured in SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes and probed with monoclonal antibody H222 (provided by Geoffrey Greene, Ben May Institute, Chicago). Complexes were detected using ECL following the manufacturer's protocol (Amersham Corp.).

RESULTS

Enhancement of ER AF-1 and AF-2 Transcriptional Activity by GRIP-1 in Yeast—GRIP-1 was originally cloned using the yeast two-hybrid system as a factor that associates with the HBD of the glucocorticoid receptor. This protein was subsequently shown to interact with and enhance the transcriptional activity of a DBD_{GAL4}-mouse ER HBD fusion protein in yeast (38). We previously developed a set of human ER mutants that contained functional AF-1 or AF-2 disruptions, and in this study investigated the transcriptional activity of these AF-selective mutants when coexpressed with the coactivator GRIP-1 in a reconstituted ER-responsive transcription assay in yeast. Yeast were chosen for this initial set of experiments because they do not appear to have any homologues of the recently identified receptor coactivators. Description of the specific ER-AF mutants is detailed in Fig. 1. Yeast were transformed with expression vectors for either wild-type estrogen receptor (wtER), ER-AF-2 (YEPE28), or ER-AF-1 (YEP3X) and a reporter plasmid containing an estrogen response element inserted upstream of the CYC1 promoter expressing the bacterial *lacZ* gene (YRPE2). The results of this analysis are shown in Fig. 2. As expected, the transcriptional activity of wtER was induced by increasing concentrations of the cognate ligand estradiol, and this activity was potentiated further by the addition of GRIP-1 (Fig. 2*A*). The maximal relative increase in transcriptional activity occurred at 0.1 nM where the coexpression of GRIP-1 resulted in a 17-fold enhancement of ER-

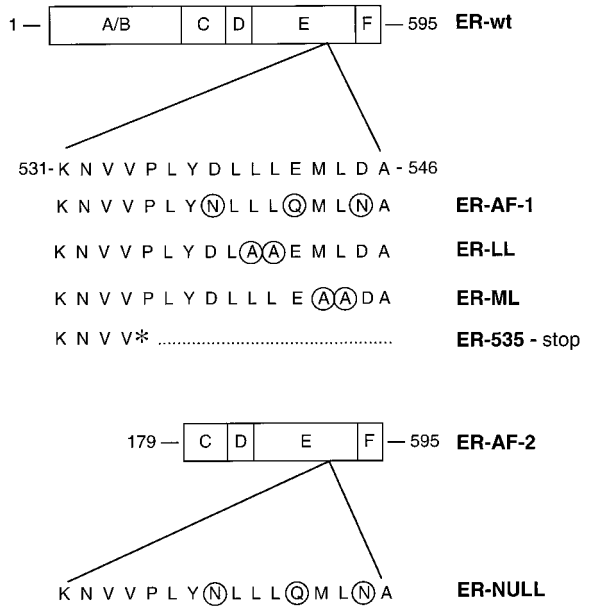

FIG. 1. **ER mutants used in this study.** A *schematic* of the wild-type ER is shown along with a region of the hormone-binding domain corresponding to AF-2. Residues that were mutated are indicated by *circles*. Also shown is a schematic of the ER AF-2 mutant receptor that lacks the N-terminal A/B domain.

dependent transcriptional activity. The transcriptional activity of the AF-2 mutant which contains amino acids 179–595 of the receptor and thus lacks the N-terminal AF-1 domain was also potentiated by the coaddition of GRIP-1 with a maximal fold induction of approximately 18 observed (Fig. 2*B*). Surprisingly, the transcriptional activity of a functional AF-2 knock-out, containing point mutations in helix 12, was also potentiated by the coactivator GRIP-1 (Fig. 2*C*). This finding was unexpected due to the fact that several reports have shown previously that coactivator binding and transcriptional enhancement is dependent on a functional AF-2 (30, 31, 38). Interestingly, the potency of estradiol-mediated transcription in the presence of GRIP-1 was increased in both the wild-type and mutant receptors as indicated by the leftward shift in the dose-response curve (Fig. 2, *A–C*). Compare the EC_{50} of wtER minus GRIP-1 at 0.5 nM with wtER plus GRIP-1 at 0.1 nM. Mutant receptors demonstrated a similar increase in potency when assayed in the presence of GRIP-1. These results suggest that the relative expression of receptor coactivators influences the efficacy of ER ligands and, in addition, indicate that a functional AF-2 is not required for coactivation by GRIP-1 in yeast.

Potentiation of the Transcriptional Activity of ER and ER AF-selective Mutants in Mammalian Cells by GRIP-1—Although yeast have proven to be a valuable tool in the dissection of the molecular mechanism of action of nuclear steroid receptors, they presumably contain no proteins homologous to the recently identified coactivators. Therefore, we felt it was necessary to extend our analysis to mammalian cells where these proteins are expressed in most if not all tissues (27, 38). We chose to perform this analysis in two distinct cell lines, the hepatocarcinoma cell line HepG2 (Fig. 3*A*) and the human cervical carcinoma cell line HeLa (Fig. 3*B*). These cell lines were chosen because we have previously shown that the known AFs operate differently in these cell types (18). AF-1 appears to be the dominant AF in HepG2 cells, whereas both AFs are required for full transcriptional activity in HeLa cells. Cells were transfected with expression vectors for either wtER, ER-AF-1 (ER TAF-1), ER-AF-2 (ER-179C), or ER-Null mutant receptors and the estrogen-responsive C3 promoter fused to the firefly luciferase reporter gene. As expected, the transcriptional activity of wtER was enhanced in the presence of estradiol in both cell lines. Further enhancement was observed when GRIP-1 was coexpressed in the cell (3-fold) although the enhancement was considerably lower than that found in yeast. Western immunoblot analysis revealed that there was no increase in ER protein levels when exogenous GRIP-1 was cotransfected (Fig. 3*C*). This demonstrates that the enhancement of ER transcriptional activity is not simply due to increased receptor levels. Interestingly, in the absence of hormone, ER transcriptional activity was slightly increased by the coaddition of GRIP-1. This appears to be the result of enhancement of unliganded ER rather than a nonspecific elevation of transcription from the C3 promoter since this activity is totally blocked by ICI182,780, an antagonist which prevents both ligand-dependent and ligand-independent transcriptional activity. Tamoxifen behaved as a partial agonist in HepG2 but not in HeLa cells as we have shown previously. However, GRIP-1 coexpression was not able to potentiate this partial agonist activity under these circumstances. Potentiation of the estrogen-dependent transcriptional activity of the AF-1 and AF-2 mutant receptors by GRIP-1 can be seen in both cell lines (4–5-fold) consistent with the observations found in yeast. GRIP-1 expression failed to potentiate the transcriptional activity of antagonist-occupied mutant receptors. Surprisingly, in HeLa cells, neither ER AF-1 nor ER AF-2 was capable of independent activity; however, exogenous GRIP-1 expression was capable of rescuing the transcriptional activity of these mutants in the presence of estradiol. GRIP-1 failed to potentiate the null receptor containing mutations in both AF-1 and AF-2. Thus, the ability of GRIP-1 to potentiate ER-AF-1 receptor but not the null receptor suggests that sequences within the ER N terminus are important for enhancement by GRIP-1 in this particular environment.

Although we have previously shown that the sequences required for estrogen responsiveness within the complement 3 (C3) promoter are comprised of three separate non-classical estrogen response elements (EREs) that act in synergy to mediate the transcriptional effects of estradiol (42), it is still possible that potentiation of ER transcriptional activity on this promoter by GRIP-1 was mediated through other factors located within the complex C3 promoter. Therefore, we decided to assay the effect of exogenous GRIP-1 expression on a simple estrogen-responsive reporter that contains only three consensus vitellogenin EREs inserted upstream of a TATA element initiating transcription of the firefly luciferase gene. In this way, we can assay transcriptional enhancement mediated solely through ER. HepG2 (Fig. 4*A*) and HeLa (Fig. 4*B*) cells were transiently transfected with a series of vectors expressing ER or ER mutants along with the 3x-ERE-TATA-Luc reporter and assayed in the presence and absence of GRIP-1. The transcriptional activity of wtER was induced upon the addition of estradiol but not the pure antagonist ICI182,780. This transcriptional activity was increased upon the addition of GRIP-1 (3.2–3.6-fold) in the presence of estradiol only. As seen on the C3 promoter, potentiation of the transcriptional activity of both ER-AF-1 and ER-AF-2 mutant receptors was evident in both cell lines with enhancement by GRIP-1 ranging from 4.1 to 9.2-fold. No additional enhancement was seen in the absence of hormone or with the antagonist ICI182,780. Interestingly, in the context of this promoter, the ER-Null receptor exhibited a weak transcriptional response that was enhanced upon the addition of estradiol and was potentiated further by the expression of GRIP-1 (2.5-fold). This suggests several possibilities; however, the two most likely explanations are as follows: 1) the point mutations introduced into helix 12 are not sufficient to

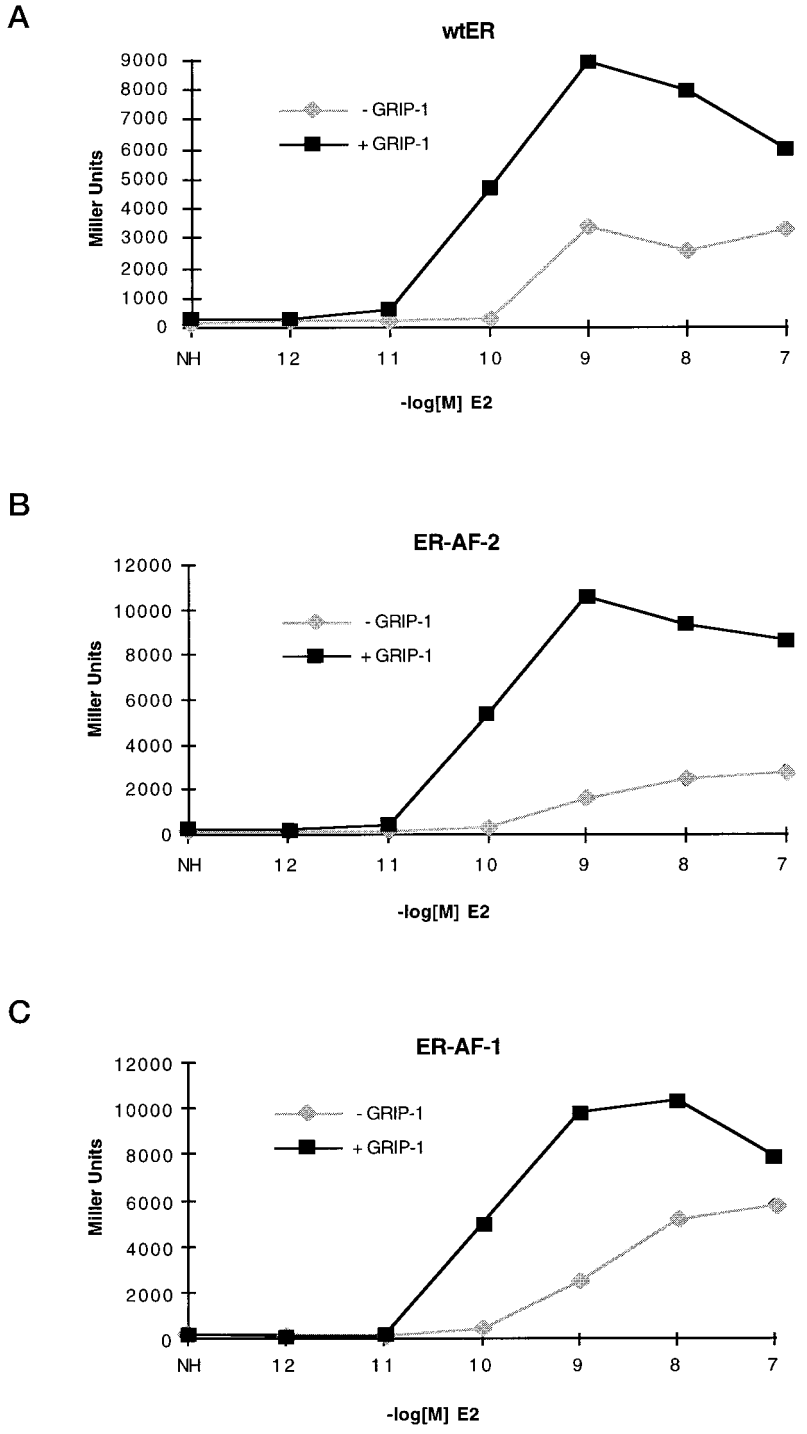

FIG. 2. **GRIP-1 enhances the transcriptional activity of both the wild-type estrogen receptor and estrogen receptor mutants in yeast.** *A*, the yeast strain YPH500 was transformed with an expression vector for the wild-type estrogen receptor along with a reporter construct containing two estrogen response elements inserted upstream of the CYC-1 promoter allowing inducible expression of the bacterial β-galactosidase gene (*YRPE2*). In addition, vectors expressing either full-length GRIP-1 (pGRIP1/fl) or no protein (pGAD424) were also transformed into yeast. Cells were induced with increasing concentrations of estradiol as indicated in the figure, and transcriptional activity was measured by assaying β-galactosidase activity. *B*, same as above except that the estrogen receptor AF-2 (YEPE28) was assessed. *C*, same as above except that the estrogen receptor AF-1 (YEP3X) was assessed.

abolish the transcriptional activity of this domain, or 2) there is another transcriptional domain within the receptor HBD that makes contacts with the coactivator GRIP-1.

Determination of the Role of AF-2 in Mediating ER Responsiveness to GRIP-1 Overexpression—To assess if GRIP-1 was interacting with regions of the receptor other than helix 12, we decided to create a series of constructs in which additional AF-2 mutations were introduced that had previously been shown to abolish not only AF-2 activity but also the transcriptional activity of AF-1. These mutations, detailed in Fig. 1, were generated by changing pairs of hydrophobic residues at amino acids 539,540 (ER-LL) and 543,544 (ER-ML) to alanines. These changes represented potentially lethal AF-2 mutants since they were previously shown to abolish the transcriptional activity of mouse ER (39). In addition, we constructed an ER in which a premature stop codon was inserted at amino acid 535 (ER-535-stop), creating a receptor mutant that lacks helix 12. HepG2 (Fig. 5*A*) and HeLa (Fig. 5*B*) cells were transfected with expression vectors containing either ER-LL, ER-ML, or ER-535-stop along with the 3x-ERE-TATA-Luc reporter and induced with a panel of estrogen receptor ligands including the agonist estradiol and the antagonists ICI182,780 and tamoxifen. Surprisingly, the transcriptional activity of these ER-AF-2 mutants were induced with all ligands tested in both cell lines. The ER-LL and ER-ML mutants displayed a robust transcriptional response to both agonist and antagonists notably evident in the HepG2 cell line. Of particular interest was the increase in transcriptional activity of the helix 12 deleted mutant upon

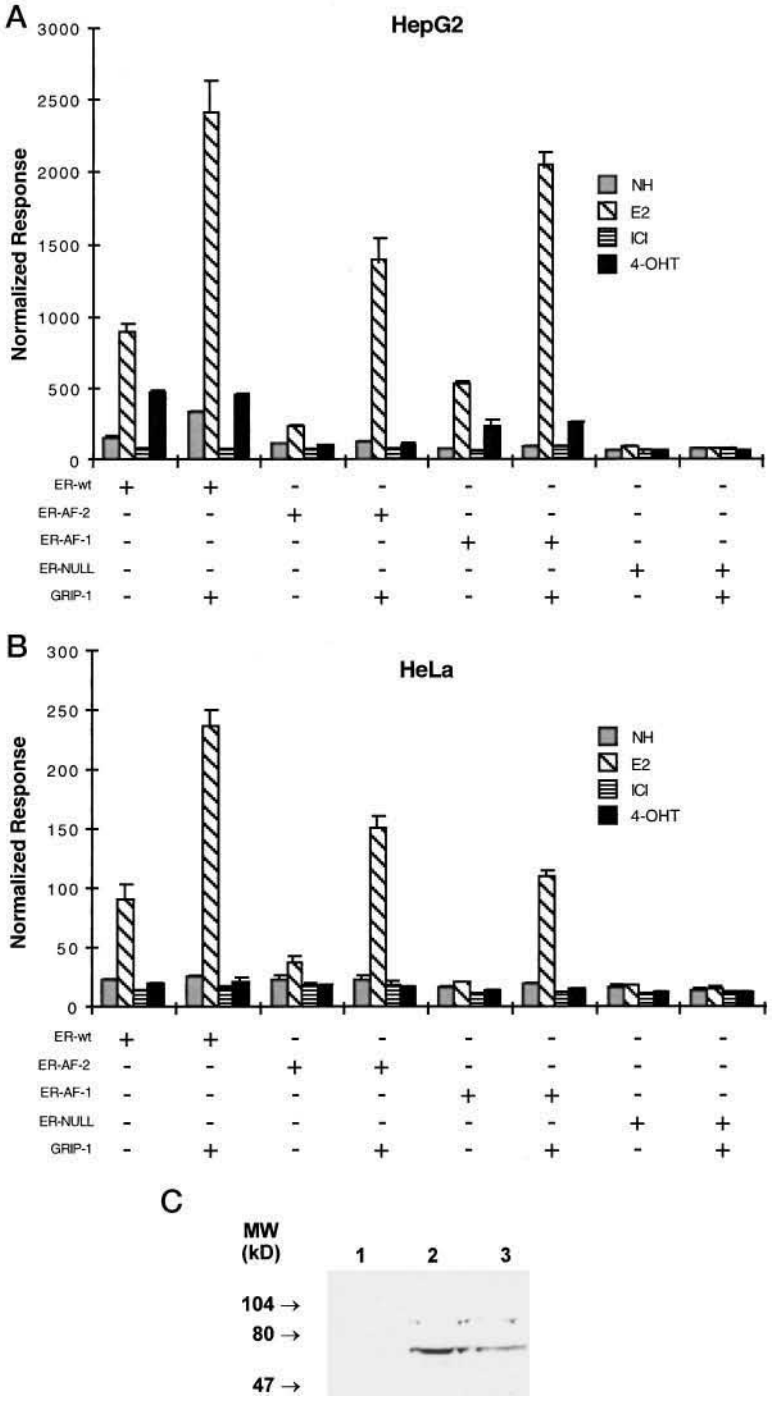

FIG. 3. **GRIP-1 enhances estrogen receptor activity on the complex estrogen-responsive C3-Luc reporter in mammalian cells.** HepG2 (*A*) or HeLa (*B*) cells were transfected with the estrogen-responsive C3-Luc reporter plasmid along with expression vectors for either the wild-type estrogen receptor, AF-2 mutant receptor, AF-1 mutant receptor, or NULL receptor. In addition, either pCMV-GRIP or an empty CMV expression vector was introduced into the cell. In addition, pCMV β-galactosidase was transfected to account for transfection efficiency. Cells were induced with ligand as indicated in the figure (100 nM). *NH*, no hormone; *E2*, 17β-estradiol; *ICI*, ICI182,780; *4-OHT,* 4-hydroxytamoxifen. Data are presented as a normalized response that was obtained by dividing the amount of luciferase activity by the β-galactosidase activity. Transfections were performed in triplicate, and error is presented as standard error of the mean. *C*, Western immunoblot analysis of nuclear extracts isolated from HeLa cells: *lane 1,* untransfected; *lane 2,* transfected with ER; *lane 3,* transfected with ER and GRIP-1. Monoclonal antibody H222 was used to detect ER protein.

the addition of ligand since previous studies failed to detect any transcriptional activity with a similar mutant ER (46). Mutation of the hydrophobic residues in helix 12 or deletion of this helix appears to disable the mechanism utilized by the receptor to distinguish between agonists and antagonists as both classes of ligands were capable of transcriptional activation. More importantly, however, is the observation that the coexpression of GRIP-1 significantly enhances this transcriptional activity, independent of the nature of the ligand bound to the receptor. Specifically, the transcriptional activities of wtER, ER-AF-1, and ER-AF-2 estrogen receptors were unaffected by GRIP-1 coexpression when analyzed in the presence of the antagonists ICI182,780 (Fig. 3), whereas the mutants ER-LL, ER-ML, and ER-535-stop were enhanced by exogenous GRIP-1 expression in the presence of both the agonist estradiol and the antagonists ICI182,780 and tamoxifen. Transcriptional potentiation by exogenous GRIP-1 expression ranged from approximately 3.5- to 10-fold, consistent with the increase in transcriptional activity measured with the other receptors tested. To determine the importance of the N terminus in mediating the transcriptional activities of ER-LL and ER-ML, we created mutations in these receptors which lacked the N-terminal 179 amino acids. The transcriptional activity of these mutant receptors was severely compromised suggesting that the N terminus is important for the unique transcriptional activities of ER-LL and ER-ML. Furthermore, exogenous GRIP-1 expression had no effect on the transcriptional activity of these mutants (data not shown). These results indicate that mutations in the AF-2 core sequence allow transactivation by ER agonists and antagonists and prevent the receptor from differentially associating

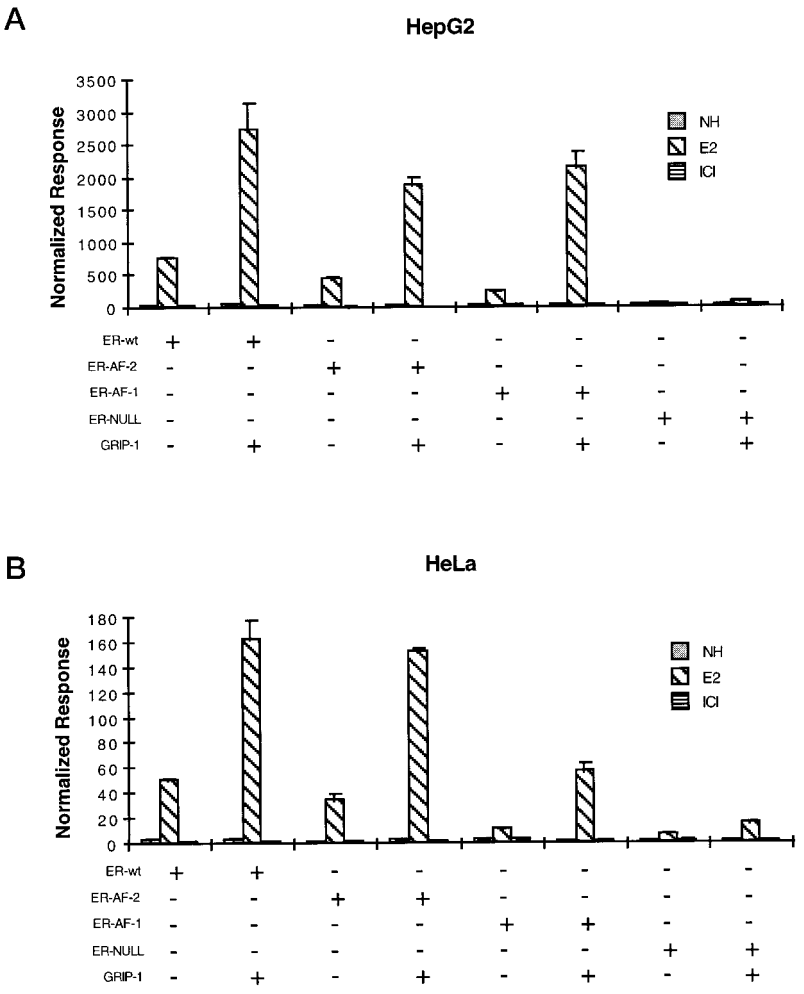

FIG. 4. **GRIP-1 enhances estrogen receptor activity on the simple 3x-ERE-TATA-Luc reporter in mammalian cells.** HepG2 (*A*) and HeLa (*B*) cells were transfected with the 3x-ERE-TATA-Luc reporter plasmid along with expression vectors for either the wild-type estrogen receptor, AF-2 mutant receptor, AF-1 mutant receptor, or NULL receptor. In addition, either pCMV-GRIP or an empty CMV expression vector was introduced into the cell. Cells were induced with ligand as indicated in the figure (100 nM). Transfection assays were normalized for transfection efficiency as described previously.

with the coactivator GRIP-1. Furthermore, these results imply that it is AF-2 activity coupled with cofactor association that determine the agonist/antagonist character of ER ligands.

GRIP-1 Fails to Enhance the Ligand-independent Transcriptional Activity of the wtER and the Mutants ER-LL and ER-ML—Previous studies have shown that ER is activated by alternate signaling pathways that do not require ligand (47). We were interested in determining the role of GRIP-1 in mediating these ligand-independent transcriptional activities. HepG2 cells were transfected with expression vectors for either the wtER, ER-LL, or ER-ML along with the 3x-ERE-TATA-Luc reporter and induced with either tamoxifen or 8-bromo-cAMP alone or in combination. All three receptors were activated by either tamoxifen or 8-bromo-cAMP alone and were further enhanced upon the addition of both compounds (Fig. 6). Surprisingly, exogenous GRIP-1 expression did not affect the transcriptional response of these receptors in the presence of 8-bromo-cAMP. As expected, GRIP-1 did enhance the transcriptional activity of the mutant receptors when bound by tamoxifen either alone or in combination with 8-bromo-cAMP. These results suggest that GRIP-1 is not capable of engaging the receptor when activated through a hormone-independent mechanism.

In Vitro Interaction Studies Reveal That the wtER but Not a Mutant Lacking AF-2 Interacts Directly with GRIP-1—Our results indicate that GRIP-1 can associate with mutant ERs in which helix 12 has been disrupted. However, the transcriptional studies utilized so far do not rule out the possibility that the interaction being measured between GRIP-1 and ER is the result of an indirect association with other ER-bound factors. We performed GST-pull-down experiments to try and determine whether these interactions were direct or indirect. A GST fusion protein containing GRIP-1 amino acids 730–1121 (38) was incubated with ^{35}S-labeled wtER or ER-535-stop. The interactions were measured in the absence of hormone and in the presence of estradiol, ICI182,780, and tamoxifen (Fig. 7). The only interaction that could be observed in this system occurred between GRIP-1 and the wtER in the presence of estradiol. No interactions could be observed with the mutant receptor and GRIP-1 or with the wtER in the absence of ligand or in the presence of antagonists. Similarly, ER-AF-1, ER-LL, and ER-ML were incapable of engaging GRIP-1 with all ligands tested (data not shown). The discrepancy observed between the results in our *in vivo* transactivation assays and *in vitro* interaction studies suggests that although GRIP-1 enhances the transcriptional activity of the ER AF-2 mutants, it is not capable of interacting with them directly. However, the possibility remains that ER contains multiple coactivator-binding sites, and the loss of AF-2 function results in a decreased affinity of ER for GRIP-1. These conditions may be unfavorable for measuring that interaction using a solution assay *in vitro* but are accommodated in the environment of the intact cell. It is also possible that the additional amino acids 322–730 contained within the pCMV-GRIP-1 construct used for transfections might contribute to the stability of the interaction between GRIP-1 and the ER mutants.

DISCUSSION

The human estrogen receptor contains three activation domains, the constitutive AF-1 located in the N-terminal region of

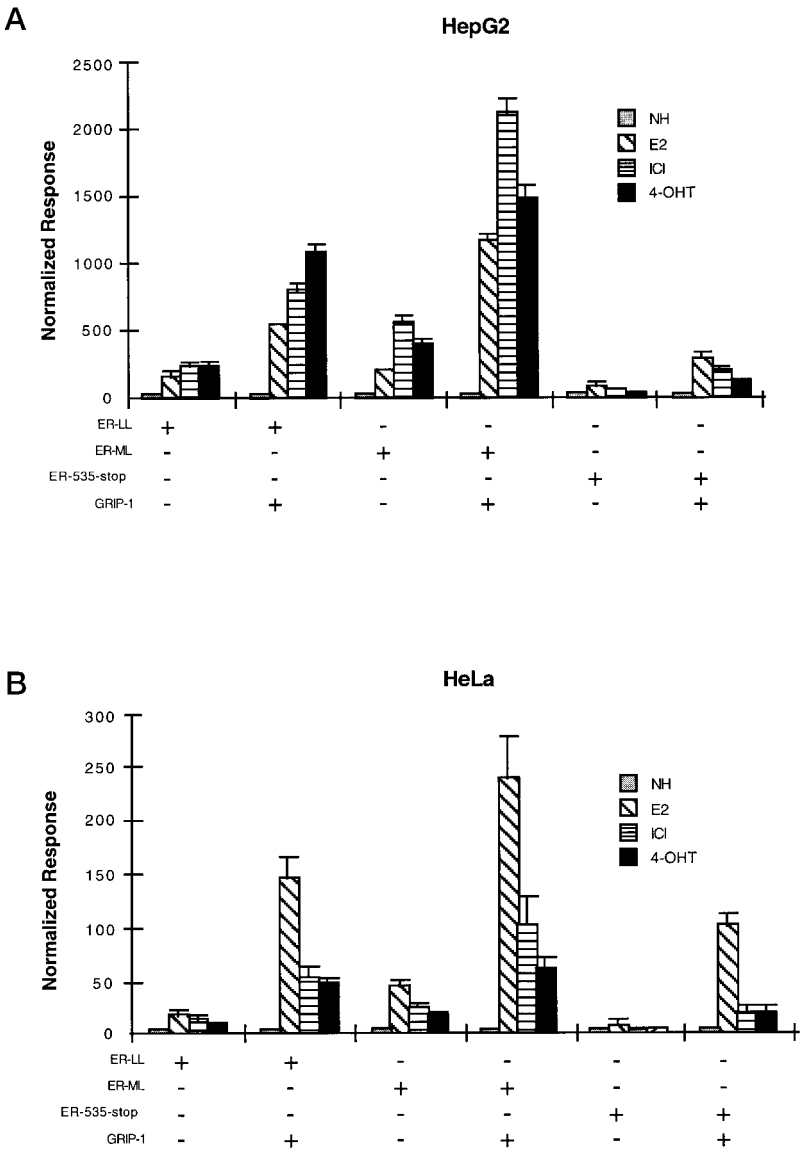

FIG. 5. **Enhancement of ER AF-2 mutants by GRIP-1.** HepG2 (*A*) and HeLa (*B*) cells were transfected with the 3x-ERE-TATA-Luc reporter along with expression vectors for either the ER-LL, ER-ML, or ER-535-stop mutant. In addition, either pCMV-GRIP or an empty CMV expression vector was introduced into the cell. Cells were induced with ligand as indicated in the figure. Transfection assays were normalized for transfection efficiency as described previously.

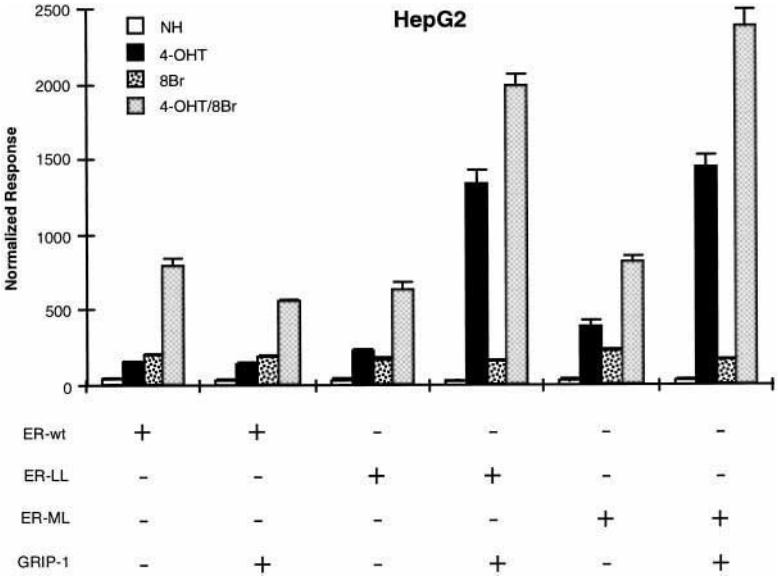

FIG. 6. **Analysis of the ligand-independent transcriptional activity of wtER, ER-LL, and ER-ML.** HepG2 cells were transfected with the 3x-ERE-TATA-Luc reporter along with expression vectors for either the wtER, ER-LL, or ER-ML receptors. In addition, either pCMV-GRIP or an empty CMV expression vector was introduced into the cell. Cells were induced with tamoxifen (100 nM) or 8-bromo-cAMP (*8Br*) (100 μM) as indicated in the figure. Transfection assays were normalized for transfection efficiency as described previously.

the receptor, the ligand-activated AF-2 positioned in the C-terminal region of the receptor, and AF2a located at the boundary between region D (hinge) and region E (hormone-binding domain). AF-1 and AF2a appear to function in a ligand-independent manner as both activation domains are capable of activating transcription in an autonomous manner (15, 17).

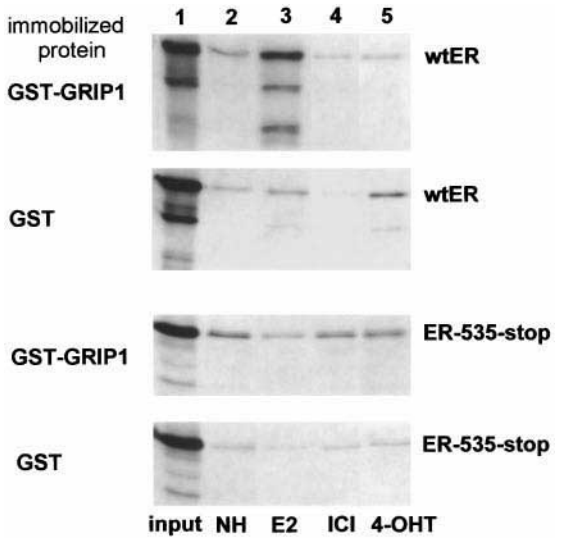

FIG. 7. **Deletion of ER helix 12 disrupts the interaction between GRIP-1 and ER *in vitro*.** GST alone or GST fusion protein, GST-GRIP-1, was isolated from bacteria and immobilized on glutathione-Sepharose beads. wtER or ER-535-stop was translated *in vitro* using rabbit reticulocyte lysate and ^{35}S-labeled methionine and incubated with the GST fusion proteins in the absence or presence of ligand as indicated in the figure (100 nM). *NH*, no hormone; *E2*, 17β-estradiol; *ICI*, ICI182,780; and *4-OHT,* 4-hydroxytamoxifen. Proteins that bound were analyzed by SDS-polyacrylamide gel electrophoresis. 1/10 input protein is included as control.

Currently, the method by which AF-1 and AF2a mediate their effects on transcription are poorly understood, although a component of the general transcriptional machinery, $TAF_{11}30$, has been shown to interact with the region identified as AF2a (48). No proteins interacting with the N-terminal region of ER or the equivalent region of other members of the nuclear receptor superfamily have been identified to date. Currently, however, a wealth of factors have been identified that interact with the HBD of several members of the nuclear receptor superfamily including ER, and it is generally believed that these proteins mediate the transcriptional effects of AF-2 located within the HBD. However, only SRC-1, GRIP-1/TIF-2 (human homologue of GRIP-1), and CBP have been shown to significantly enhance transcription, and consequently they are the only factors thus far that can be considered bona fide coactivators for nuclear receptors (27, 28, 38, 49, 50). SRC-1 and GRIP-1/TIF-2 belong to the same family of proteins, and CBP belongs to a separate family of proteins that includes p300 (28, 51). Our previous studies demonstrated that AF-2 was an important component of the ability of the receptor to distinguish between agonists and antagonists. The good correlation between AF-2 function and the ability of coactivators to exhibit a positive influence on ER transcription suggests, therefore, that these interactions are an integral part of this discrimination.

GRIP-1 Enhances Agonist but Not Antagonist-mediated ER Transcriptional Activity—By using a series of experiments designed to examine the effects of GRIP-1 expression on the transcriptional properties of ER, we find that in the context of the wtER exogenous GRIP-1 significantly enhances estradiol-mediated transcription and fails to potentiate the activity of antagonist-occupied receptors including the partial agonist activity of tamoxifen. Exogenous GRIP-1 does not alter the expression level of ER, ruling out the possibility that increased receptor levels are responsible for the increase in transcriptional activity. Enhancement by GRIP-1 appears to be independent of cell type as similar results were observed in the following two distinct cell lines: HepG2 in which the mixed agonist tamoxifen activates ER, and HeLa in which tamoxifen fails to mediate transcription and thus behaves as a complete antagonist. This is in agreement with several lines of evidence which suggest that the partial agonist activities imparted by mixed agonists are mediated by the N-terminal (AF-1) activation domain (14). Interestingly, the highly related coactivator, SRC-1, has been shown to enhance not only estradiol-mediated transcription but also the mixed agonist activity of tamoxifen (50). It was proposed that a hormone-independent mechanism existed to promote the association of SRC-1 with ER since SRC-1 could enhance hormone-independent ER transcriptional activity in the presence of forskolin and 3-isobutyl-1-methylxanthine (50). These compounds have been linked to the ability of tamoxifen to switch from an anti-estrogen to a partial estrogen in certain cell types (46, 47). Thus, it appears that SRC-1 is capable of engaging ER in contexts where estradiol is not required. Our results failed to demonstrate an enhancement of either tamoxifen-activated or ligand-independent ER transcriptional activity and suggest that there is a divergence in the biocharacter of GRIP-1 and SRC-1 when associated with antagonists bound or ligand-independent activated ER.

GRIP-1 Enhances ER-mediated Transcription in the Absence of a Functional AF-2—The ability of GRIP-1 to enhance the transcriptional activity of a series of ER mutants in which helix 12 (AF-2) has been disrupted suggests that sequences in addition to helix 12, located in the HBD or sequences in the N terminus of the receptor, contribute to coactivator association. Our data imply that, depending on cell and promoter context, both may be correct. Specifically, we noted that the requirement for the N terminus appears to be promoter-specific as this domain is required for enhancement by GRIP-1 on the C3-Luc promoter but not the 3x-ERE-TATA-Luc promoter. The most remarkable example of GRIP-1-mediated enhancement is on the C3-Luc reporter in HeLa cells where its expression can rescue transcriptionally inactive mutants in which either AF-1 or AF-2 have been disrupted. This activity is not restricted to cell or promoter contexts as the same activity is observed in HepG2 cells or with a simple promoter containing only estrogen response elements and a TATA sequence element. Transcriptional enhancement of ER-AF-2 mutants by GRIP-1 indicates that this coactivator is capable of interacting with an ER mutant previously thought to be transcriptionally silent. Furthermore, GRIP-1 is able to activate a mutant ER in which helix 12 has been deleted. This strongly suggests that the GRIP-1 interaction surface does not depend solely on the integrity of the AF-2 core domain but instead is composed of distinct receptor regions that coalesce upon ligand binding. These findings are consistent with the inability of ER helix 12 to mediate transcription independently (52) and the known crystal structures of holo-RXRα (20) and apo-RARγ (21) which show that a major reorganization of the HBD occurs in the presence of ligand. These findings are also consistent with the work of others (53) who have shown using GST fusion protein pull-down assays that SRC-1 can associate with ER in the absence of helix 12. Additionally, a lysine residue located in helix 3 which is highly conserved among members of the nuclear receptor superfamily has been shown to be important for coactivator association (54). It is interesting to note that helix 12 comes into close proximity to helices 3 and 4 when bound by ligand suggesting that these helices together may form the surface for which receptor-associated proteins interact.

The ER AF-2 Domain Functions as a Discriminator between Agonists and Antagonists—Our previous data and that presented here suggest that one of the functions of the AF-2 domain is to allow the discrimination between different classes of ligands. When select point mutations are introduced into AF-2, the receptor loses the ability to distinguish between

agonists and antagonists. Specifically, we observed that the ER-LL, ER-ML, and ER-535-stop receptors are activated by the agonist estradiol, the complete antagonist ICI182,780, as well as the mixed agonist tamoxifen. Interestingly, these mutants are also able to interact with and are enhanced by coexpression of GRIP-1, regardless of the ligand used to activate the receptor. This finding is similar to a report which demonstrated that mutations introduced into helix 12, which attenuate ER transcriptional activity, facilitated antagonist-induced activation when assayed in the presence of 3-isobutyl-1-methylxanthine and cholera toxin (46). These agents lead to a marked increase in intracellular cAMP levels and are thought to initiate or facilitate intracellular signal transduction pathways. Therefore, it would be intriguing to speculate that phosphorylation of ER or cellular factors like GRIP-1 might enhance the interaction between these two proteins and allow antagonists to function as agonists. Previously, mutation of hydrophobic residues in the mouse estrogen receptor AF-2 was shown to permit transcriptional activation by the antagonists ICI182,780 and tamoxifen. However, unlike human ER, the agonist estradiol behaved as a complete antagonist (55). Thus, in the mouse ER, mutation of the hydrophobic residues in AF-2 switched the transcriptional response of agonist- and antagonist-occupied receptors, whereas in the human ER, mutations in AF-2 resulted in the loss of discrimination between the different classes of ligands. Perhaps these mutations in the mouse ER allow antagonists to recruit the coactivator GRIP-1, or a similar factor, resulting in ER transcriptional activity.

It has been proposed that mutations in the AF-2 core domain of ER could be important clinically in that it would provide a mechanism by which ER-responsive tumors could become refractory to antihormone treatment (55). However, it is unclear how important these mutants are in regulating physiological target genes as their overall transcriptional activity has been attenuated. The observations in this paper would suggest that alterations in the expression of receptor coactivators can convert these AF-2 mutant receptors into potent transcriptional activators. In this regard, a novel nuclear receptor coactivator, AIB1, was found to be overexpressed in primary breast cancer cells as well as in primary breast tumors (56). The transcriptional activity of the AF-2 mutant ERs is similar to the activity of a C-terminal truncated progesterone receptor (PR) mutant that was shown to be activated by the PR antagonist RU486 (57). At the time, it was postulated that this activation by RU486 was mediated by the loss of a C-terminal repressor that is associated with PR in the presence of antagonist. Further studies established that a peptide encompassing the C-terminal region of PR demonstrated transcriptional silencing activity when fused to the GAL4 DNA-binding domain (58). The results presented in this study would suggest that, in addition to the loss of a putative corepressor, truncation of the C terminus might allow RU486 to recruit coactivators like GRIP-1, thus permitting transcriptional activation. Interestingly, we have previously shown that another mutation in the AF-2 core domain, specifically D538N/E542Q/D545N (ER-AF-1), was activated by the antagonist raloxifene, the mixed agonists tamoxifen, and estradiol but not the complete antagonist ICI182,780 (18). This suggests that specific mutations in AF-2 could result in mutant receptors that are activated by a specific class of ligand. Thus, it is probable that different classes of anti-estrogens that are known to induce unique conformational states within the HBD may have utility in the treatment of ER-positive breast cancers which become refractory to initial endocrine therapy.

The mechanism by which coactivators enhance transcription is to date poorly understood. Possibilities include stabilization of the protein as a consequence of structural alterations in the hormone-binding domain, enhancement of DNA binding, and interaction with other factors which would contribute to the stability of the general transcription machinery. It is unlikely that GRIP-1 stabilizes receptor levels as we did not observe differences in the expression level of ER in the presence or absence of exogenous GRIP-1 in mammalian cells. Furthermore, enhancement of glucocorticoid receptor activity in yeast by GRIP-1 was shown to occur in the absence of increased glucocorticoid receptor protein levels (38). Our data suggest, depending on the cell and promoter context, that both N-terminal (AF-1) and C-terminal (AF-2) sequences are important for potentiation of ER transcriptional activity by GRIP-1. Enhancement of ER mutants by GRIP-1 in which either AF-1 or AF-2 has been disrupted can be demonstrated with the resultant transcriptional response ultimately depending on the cell type and the relative level of coactivators. However, when both AFs are disrupted, GRIP-1 promotes only a marginal transcriptional enhancement of ER activity. This suggests that GRIP-1 association is dependent on several distinct receptor regions, including the N terminus, that upon ligand binding form a competent surface for interaction. It is not yet known whether amino acid residues within these regions physically contact GRIP-1 or are important for maintaining the appropriate conformation that GRIP-1 recognizes. In any event, the method by which this interaction mediates transcription remains to be elucidated. It is likely, however, that the coactivators engage additional cellular components, which as a complex are capable of initiating ER transcriptional activation. Specifically, SRC-1 has been shown to interact with CBP, which, in addition to having intrinsic histone acetylase activity (59, 60), contacts TATA box binding protein and TFIIB, members of the general transcription apparatus (61, 62). Histone acetylation is thought to facilitate chromatin remodeling by modifying core histones. Thus, coactivators may help to stabilize the interaction between CBP and ER resulting in a local change in the chromatin environment with a subsequent enhancement in recruitment of the RNA polymerase II complex. Therefore, CBP would act as a bridge between the nuclear receptor-coactivator complex and the general transcription apparatus. It is also possible that the coactivators stabilize the general transcription machinery directly as SRC-1 has been shown to interact with TATA box binding protein and TF11D (53). In conclusion, our results highlight the complex nature of coactivator association with ER and provide evidence that this interaction is a major determinant of the pharmacology of ER ligands. Ultimately, it will be interesting to see if GRIP-1/TIF-2, SRC-1, CBP, and other factors shown to enhance transcriptional activity through the AF-2 region can interact with the nuclear receptors simultaneously and if these factors make selective contacts with members of the general transcription machinery as well as with each other.

Acknowledgment—We thank Kulwant Kohli for technical assistance.

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