Estradiol repression of tumor necrosis factor-α transcription requires estrogen receptor activation function-2 and is enhanced by coactivators

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The tumor necrosis factor-α (TNF-α) promoter was used to explore the molecular mechanisms of estradiol (E_2)-dependent repression of gene transcription. E_2 inhibited basal activity and abolished TNF-α activation of the TNF-α promoter. The E_2-inhibitory element was mapped to the −125 to −82 region of the TNF-α promoter, known as the TNF-responsive element (TNF-RE). An AP-1-like site in the TNF-RE is essential for repression activity. Estrogen receptor (ER) β is more potent than ERα at repressing the −1044 TNF-α promoter and the TNF-RE upstream of the herpes simplex virus thymidine kinase promoter, but weaker at activating transcription through an estrogen response element. The activation function-2 (AF-2) surface in the ligand-binding domain is required for repression, because anti-estrogens and AF-2 mutations impair repression. The requirement of the AF-2 surface for repression is probably due to its capacity to recruit p160 coactivators or related coregulators, because overexpressing the coactivator glucocorticoid receptor interacting protein-1 enhances repression, whereas a glucocorticoid receptor interacting protein-1 mutant unable to interact with the AF-2 surface is ineffective. Furthermore, receptor interacting protein 140 prevents repression by ERβ, probably by interacting with the AF-2 surface and blocking the binding of endogenous coactivators. These studies demonstrate that E_2-mediated repression requires the AF-2 surface and the participation of coactivators or other coregulatory proteins.

Estrogens exert profound effects on bones, the cardiovascular system, the urogenital tract, and the nervous system in women (1–3). Estrogen replacement in postmenopausal women reduces hot flashes and the risk of osteoporosis (4), cardiovascular disease (5), and Alzheimer's disease (6). Despite these benefits, many eligible women decline to take estrogens because of their adverse effects, such as the increased risk of breast and endometrial cancer (7). Recently, the tissue-selective estrogen receptor modulator raloxifene has been introduced as an alternative to estrogens to minimize the adverse effects of hormone replacement (8, 9). Raloxifene enhances bone mineral density (10) and is used clinically to prevent osteoporosis (11). Unlike estrogens, raloxifene does not stimulate endometrial growth (12) and prevents breast cancer (13). Despite these important effects, raloxifene and other selective estrogen receptor modulators are less effective at increasing bone mineral density (10) and ineffective at alleviating hot flashes (8, 9), and they may not provide other benefits of estrogens, such as the reduction in cardiovascular disease (14). Whereas studies with raloxifene demonstrate that it is possible to remove the adverse effects of estrogens, while retaining at least some beneficial effects, it is important to develop better drugs for hormone replacement so that women can reap the full benefits of estrogens.

Development of better estrogens for hormone replacement requires a more complete understanding of the molecular mechanisms of how estrogens regulate gene transcription. Estrogens activate or repress gene transcription by binding to two distinct estrogen receptors (ERs), ERα and ERβ (15, 16). Estrogen activation of gene expression has been studied extensively. The activation of gene transcription by estrogens requires ER dimerization, ER binding to an estrogen response element (ERE), activation function-1 (AF-1) in the A/B domain, activation function-2 (AF-2) in the ligand-binding domain (LBD), and coactivator proteins (15, 16). In contrast, the ER region and cofactors involved in estradiol (E_2)-dependent repression are unknown. It is especially important to understand how ERs mediate repression of specific genes in bone, because estrogens prevent osteoporosis by inhibiting bone resorption (1–3).

Estrogens decrease production of tumor necrosis factor-α (TNF-α), which is associated with the pathogenesis of osteoporosis (17–19). TNF-α levels rise with the drop in E_2 levels after oophorectomy in premenopausal women and estrogen replacement after surgery reduces TNF-α levels to baseline (20). Furthermore, E_2 decreases TNF-α production in peripheral monocytes from postmenopausal women (21) and TNF-α mRNA in the human monocytic THP-1 cell line (22). These studies suggest that estrogens may inhibit TNF-α production by repressing TNF-α gene transcription. Thus, in this study we selected the TNF-α promoter to explore molecular mechanisms of how estrogens repress gene transcription. We show that E_2 inhibits basal activity and TNF-α induction of its own promoter and that ERβ is more potent than ERα at mediating repression. We further demonstrate that E_2-mediated repression requires the AF-2 surface and is enhanced by coactivator proteins, which were previously shown to mediate positive responses of nuclear receptors.

Materials and Methods

Reagents. Phenol red-free Dulbecco's modified Eagle's/F-12 Coon's modification medium was obtained from Sigma. Biobrene was purchased from Applied Biosystems. The U937 cell line was obtained from American Type Culture Collection. Human recombinant TNF-α was obtained from R & D Systems.

Plasmid Construction. A *Pst*I to *Aha*II fragment (−1044 to +93) from the human TNF-α gene, *pLT*, was cloned upstream of the luciferase cDNA. The 5′ deletions were constructed by using unique restriction sites, *Apa*I for the −125 deletion, and *Sty*I for the −82 deletion, as previously described (23). Three copies of the human TNF-α promoter fragment from −125 to −82 (23) [TNF-responsive element (TNF-RE)] or one copy of the ERE from the frog vitellogenin *A2* gene (vitA2-ERE, 5′-TCAGGTCACAGTGACCTGA-3′) were ligated upstream of −32 to +45 herpes simplex thymidine kinase (TK) promoter linked to luciferase (TNF-

Abbreviations: AF, activation function; DBD, DNA-binding domain; E_2, estradiol; ER, estrogen receptor; ERE, estrogen response element; GRIP, glucocorticoid receptor-interacting protein; LBD, ligand-binding domain; Luc, luciferase; NR, nuclear receptor; RIP140, receptor interacting protein 140; TNF-α, tumor necrosis factor-α; TNF-RE, TNF-responsive element; TK, thymidine kinase; ICI, Imperial Chemical Industries.

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RE TKLuc, and ERE TKLuc, respectively). ERβ mutants were created with QuikChange site-directed mutagenesis kits (Stratagene), by using oligonucleotides containing the mutation. The mutants were sequenced with Sequenase kits (Amersham Pharmacia) to verify the presence of the mutation. Expression vectors for human ERα, human ERβ485, glucocorticoid receptor interacting protein-1 (GRIP1), GRIP Nuclear Receptor (NR) box II/III mutant, and RIP140 were previously described (24).

Cell Culture and Transfection. U937 cells were maintained and subcultured in phenol red-free DME/F-12 Coon's Modification medium containing 10% newborn bovine serum/2 mM glutamine/50 units/ml penicillin/50 μg/ml streptomycin. Cells were collected by centrifugation and then resuspended in Dulbecco's PBS (0.5 ml/1.5 × 10^7 cells) containing 0.1% dextrose/10 μg/ml Biobrene, a luciferase reporter plasmid, and a plasmid that expresses human ERα or ERβ. The cells were transferred to a cuvette and then electroporated by using a Bio-Rad gene pulser as previously described (23). After electroporation the cells were transferred to new medium and plated at 1 ml per dish in 12-well multiplates. Cells were treated with 17 β–E_2 for 3 h before exposure to 5 ng/ml TNF-α. After 24 h at 37°C, cells were collected by centrifugation, lysed by the addition of 200 μl 1× lysis buffer (Promega), and assayed for luciferase activity with a kit (Promega). The concentration of E_2 that is required for half-maximal induction (EC_{50}) or inhibition (IC_{50}) of luciferase activity was calculated with the PRISM curve-fitting program (GraphPad, San Diego). All transfection studies were performed at least three times. Each point represents the mean of triplicate samples +/− SEM.

Electrophoretic Mobility-Shift Assay. The TNF-RE spanning the −125 to −82 region of the TNF-α promoter and the vitA2-ERE were end labeled by 5′ phosphorylation with T4 polynucleotide kinase and [γ-^{32}P]ATP. DNA-binding reactions were performed in 20 μl containing ^{32}P-labeled TNF-RE or ERE and in final concentration 12 mM Hepes-KOH, pH 7.6/48 mM KCL/0.8 mM EDTA/4 mM MgCl2/10% glycerol/0.05% Nonidet P-40/2 μg of dI-dC. The binding reaction was initiated by the addition of 2 μl of ERα or ERβ that had been prepared with an *in vitro* transcription/translation kit (Promega) or of U937 cell nuclear extract prepared as previously described (23). The samples were incubated for 15 min at room temperature, placed on ice, and then loaded on a 5% nondenaturing polyacrylamide gel. The samples were electrophoresed at 200 mV with running buffer consisting of 25 mM Tris base/25 mM borate/1 mM EDTA.

Results

E_2 Inhibits TNF-α Activation of the TNF-α Promoter. To test our hypothesis that E_2 decreases TNF-α production by repressing TNF-α gene transcription, we examined the effect of E_2 on the TNF-α promoter in human monocytic U937 cells. We used these cells because they synthesize and secrete TNF-α and are used extensively to study the regulation of TNF-α production (23, 25). Like most cultured cell lines, these cells do not have detectable levels of ERs and require transfection of ERs to elicit effects on transcription (data not shown). TNF-α stimulated the −1044 TNF-α, and −125 TNF-α promoters by two- to threefold in the absence of E_2 (Fig. 1 *A* and *B*). E_2 inhibited basal activity and abolished TNF-α activation of these promoters in the presence of transfected ERα (Fig. 1*A*) and ERβ (Fig. 1*B*). No TNF-α activation or E_2 repression was observed with the −82 TNF-α promoter. These results demonstrate that the E_2-inhibitory element is located in the −125-to-−82 region, known as the TNF-RE (23).

ERβ Is More Potent Than ERα at Repression, but Weaker at Activating at an ERE. We examined whether ER isoforms inhibit TNF-α activation of the TNF-RE in the context of a heterologous promoter. Whereas the initial human ERβ cDNA encoded for a protein that contains 485 amino acids (ERβ485) (26, 27), a longer cDNA has been isolated, which contains 45 additional amino acids in the N-terminal region (530 amino acids, ERβ530) (28). Because the relative functional roles of these isoforms are not known, we compared ERβ485 and ERβ530 with ERα for repression and activation activity. TNF-α produced a 5- to 10-fold activation of three copies of the TNF-RE (−125 to −82) upstream of the minimal TK (TNF-RE TKLuc) promoter. E_2 repressed TNF-α activation by 60–80% in the presence of all ERs (Fig. 1*C*). However, ERβ485 and ERβ530 were approximately 20-fold more potent than ERα at repression (IC_{50} of 241 pM for ERα vs. 13 pM and 15 pM for ERβ485 and ERβ530, respectively). In contrast, ERα produced a 10-fold-greater activation of the classical ERE compared with ERβ (Fig. 1*D*), but all three EC_{50} values were similar (data not shown). Thus, the long and short ERβs are more potent than ERα at repression, even though they are less effective than ERα at activating an ERE. Furthermore, no differences in activation or repression activity were observed for ERβ485 and ERβ530, suggesting that the functional activity of ERβ is similar, regardless of which form is expressed in cells. ERβ485 is also more potent than ERα at repressing the −1044 TNF-α promoter (Fig. 2*A*).

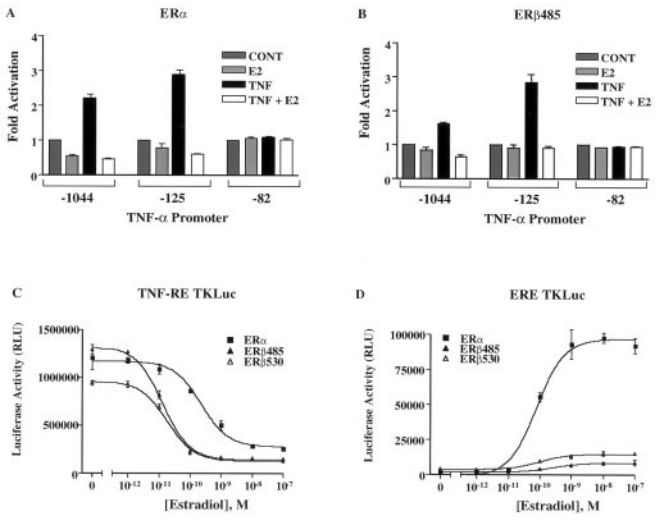

Fig. 1. The −125 to −82 region of the TNF-α promoter is required for E_2 repression. A deletion series of the human TNF-α promoter (3 μg) was transiently transfected individually into U937 cells with 1 μg of expression vector for human ERα (*A*) or ERβ485 (*B*). Cells were treated for 24 h with TNF-α (5 ng/ml) in the absence or presence of 10 nM E_2, and luciferase activity was measured. (*C*) ERβ is more potent than ERα at repressing TNF-α activation of the TNF-RE. U937 cells were transfected with 3 μg of TNF-RE TKLuc and 1 μg of human ERα, ERβ485, or ERβ530. Cells were treated for 24 h with TNF-α (5 ng/ml) in the presence of increasing concentrations of E_2, and luciferase activity was measured. (*D*) ERα is more effective than ERβ at activating an ERE. U937 cells were transfected with 3 μg of ERE TKLuc and 1 μg of human ERα, ERβ485, or ERβ530. Cells were maintained in the absence or presence of 10 nM E_2 for 24 h and then assayed for luciferase activity.

An AP-1-Like Site Is Required for Repression. The −125 to −82 region contains an AP-1/cyclic AMP response-like element (5′-TGAGCTCA-3′) that binds c-jun (23), and is flanked by ETS, CCAAT/enhancer-binding β protein, nuclear factor of activated T cells, and NF-κB elements (29–32). The AP-1 site is essential for TNF-α activation and E_2 repression, because both activities are abolished if this site is mutated (data not shown). We previously reported that E_2 activates transcription of an AP-1 element in the collagenase promoter in other cell types (33), suggesting that the differences in response to E_2 may be related to the cell type or promoter sequence. To test this hypothesis, the collagenase pro-

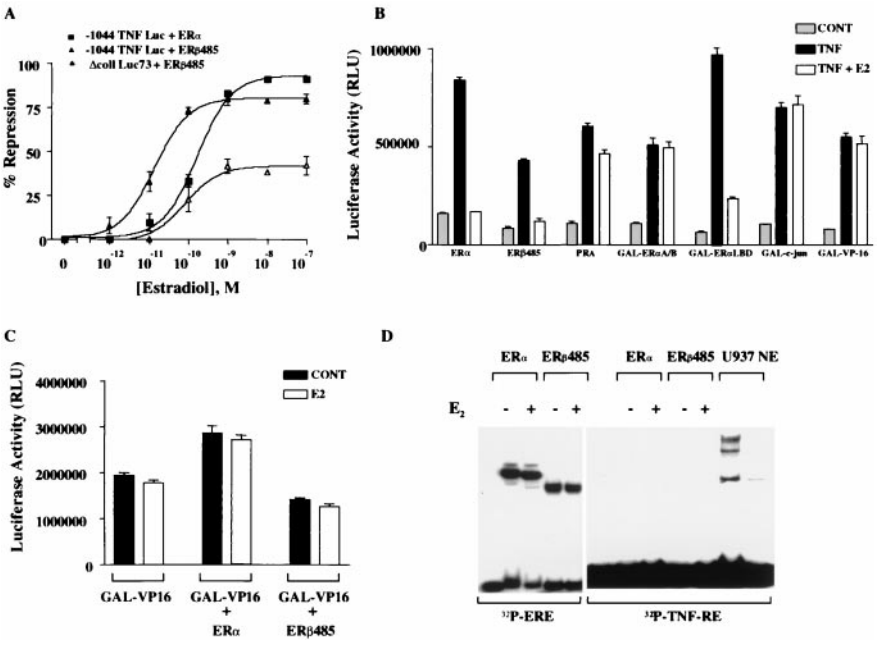

Fig. 2. E_2 represses the collagenase promoter in U937 cells. (*A*) Cells were transfected with 3 μg of -1044 TNF-α Luc or the AP1-driven collagenase luciferase reporter (Δcoll73) plasmid (33) and 1 μg of human ERα or ERβ485. After transfection, the cells were treated for 24 h with TNF-α (5 ng/ml) in the presence of increasing concentrations of E_2, and luciferase activity was measured. (*B*) The ERα LBD contains the repression domain. U937 cells were transfected with 3 μg of TNF-RE TKLuc and 1 μg of human ERα, ERβ485, human progesterone receptor A (PRA), GAL-ERα A/B domain, GAL-ERα LBD, GAL-c-jun, or GAL-VP16. Cells were treated for 24 h with TNF-α (5 ng/ml) in the presence 10 nM E_2 or 100 nM progesterone and then assayed for luciferase activity. (*C*) ERα and ERβ do not inhibit GAL-VP16 activation of $GALRE_5$ Luc. Cells were transfected with 3 μg of GAL-responsive element-5 (RE_5) Luc (pG5-Luc, Promega), 1 μg of GAL-VP16, and 1 μg of either ERα or ERβ485. After 24 h treatment with 10 nM E_2, cell extracts were assayed for luciferase activity. (*D*) ERs do not bind to the TNF-RE. Electrophoretic mobility-shift assays were performed by using ^{32}P-labeled ERE or TNF-RE probes with 2 μl of *in vitro*-transcribed and -translated ERα or ERβ485. Binding was performed in the absence ($-$) or presence ($+$) of 10 nM E_2. U937 cell nuclear extracts (NE) were prepared (23) and incubated with ^{32}P-labeled TNF-RE in the absence or presence of 100 ng of unlabeled TNF-RE (last lane) as described in *Materials and Methods*.

moter was cotransfected with ERβ485 into U937 cells. In contrast to other cell types (33), E_2 also repressed the collagenase promoter in U937 cells. However, the potency and the magnitude (35%) of repression of the collagenase promoter by E_2 are less than ERα- and ERβ485-mediated repression of the -1044 TNF-α (80%) promoter (Fig. 2*A*). These results demonstrate that E_2 stimulates or represses gene transcription at AP-1 sites in a tissue-specific fashion. More importantly, these observations suggest that the arrangement of transcriptional factors along the TNF-RE is important for E_2 repression.

The Repression Domain Is Present in the LBD. To map the repression domain in ERα, the TNF-RE TKLuc was cotransfected with ERα deletion mutants and chimerical proteins (Fig. 2*B*). E_2 repression persisted with an ERα that lacked the amino terminal A/B domain or the DNA-binding domain (DBD; data not shown). A chimerical ER consisting of the ERα LBD fused to the GAL DBD produced repression activity similar to the full-length ERα (Fig. 2*B*). In contrast, chimerical proteins consisting of the GAL DBD fused to the ERα A/B domain, c-jun, or VP16 were ineffective at repressing TNF-RE TKLuc (Fig. 2*B*), demonstrating that the repression function is specific to the ER LBD and is not mediated by the GAL DBD. Overexpression of progesterone receptor A exhibited much less repression activity (20%) compared with ERα and ERβ485 (Fig. 2*B*). Furthermore, ERα and ERβ485 did not significantly inhibit the activation of GAL-responsive element by GAL-VP16 (Fig. 2*C*). These results suggest that repression by ERs does not occur simply by squelching limiting amounts of transcriptional factors.

ERs Do Not Bind Directly to the TNF-RE. To determine whether ERs bind to the TNF-RE, we performed gel shift assays by using *in vitro*-translated ERα and ERβ485. As expected the ERs bound to the vitA2-ERE (Fig. 2*D*). In contrast, ERα and ERβ485 did not bind to TNF-RE, which is consistent with our functional studies showing that the ER DBD is dispensable for repression. However, specific binding of factors to the TNF-RE was observed with nuclear extracts prepared from U937 cells (Fig. 2*D*). These studies suggest that ER mediates repression via protein-protein interactions, rather than directly binding to the TNF-RE.

Anti-Estrogens Antagonize Repression by E_2. We explored the role of the AF-2 surface in repression, because the repression domain mapped to the LBD and the AF-2 surface is required for E_2 activation of transcription at a classical ERE. Helices 3, 5, and 12 form the AF-2 surface (34–37), which interacts with the p160 class of coactivators, such as steroid receptor coactivator-1 and GRIP1 (38, 39). By contrast, the ER receptor surface that mediates repression is not known, nor are the factors that interact with ER to trigger repression. The role of the AF-2 surface in repression was investigated by determining the effects of anti-estrogens on repression, because they prevent the formation of an active AF-2 surface (34, 36). In contrast to repression observed with E_2, the anti-estrogens raloxifene, tamoxifen, and ICI 182,780 produced a weak dose-dependent enhancement (approximately twofold) of TNF-α activation of the TNF-RE in the presence of transfected ERα (Fig. 3*A*) and ERβ485 (Fig. 3*B*). Raloxifene also increased TNF-α activation of the -1044 TNF promoter by two- to sixfold (data not shown). In contrast, raloxifene, tamoxifen, and Imperial Chemical Industries (ICI) 182,780 exhibited a dose-dependent antagonism of E_2 inhibition of the TNF-α activation with ERα and ERβ485 (Fig. 3 *C* and *D*, respectively). Thus, similar to their effects at an ERE, the anti-estrogens act as antagonists at the TNF-RE, most likely by preventing formation of an active AF-2 surface. These results suggest that the AF-2 surface in the LBD mediates repression of the TNF-α promoter by ERs, and they confirm our previous observation that anti-estrogens require other regions of ER for their effects (24, 33).

Mutations in the AF-2 Surface Block Repression. To further explore the role of the AF-2 surface in repression, point mutations were introduced in helix 3 (K269A) and helix 12 (E448K) of the AF-2 surface of ERβ485 based on the ERα LBD x-ray structure (34). Mutations of homologous amino acids in these two helices of ERα (35, 40) and other nuclear receptors (35) are the most disruptive to the formation of an active AF-2 surface required for activation. Similar to our previous results with ERα (35), the ERβ485 AF-2 mutants were still capable of binding to an ERE, but unable to bind to GRIP1 and mediate E_2 activation of ERE TKLuc (data not shown). ERβ485 AF-2 mutants showed a profound decrease in repression activity of the TNF-RE (Fig. 4*A*) and -1044 TNF-α

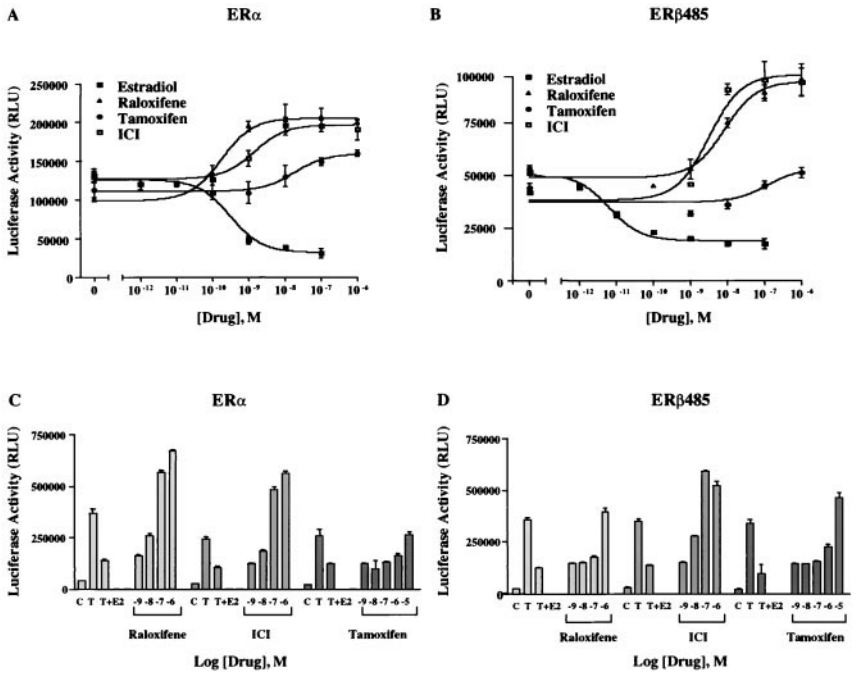

Fig. 3. Anti-estrogens do not inhibit TNF-α activation of the TNF-RE, but block E_2 repression of TNF-α activation. U937 cells were transfected with 3 μg of TNF-RE TKLuc and 1 μg of human ERα (*A* and *C*) or human ERβ485 (*B* and *D*). U937 cells were treated for 24 h with TNF-α (5 ng/ml) in the presence of increasing concentrations of E_2, raloxifene, tamoxifen, or ICI (*A* and *B*). In *C* and *D*, all cells were treated for 24 h with TNF-α (T) and 1 nM E_2 (T + E2) in the presence of increasing concentrations of raloxifene, ICI, or tamoxifen, except for the control (C) cells.

promoter (data not shown), whereas mutations in other putative ERβ helices had little effect on repression (data not shown). Thus, the region required for repression is the same or overlaps with the AF-2 surface necessary for coactivator binding and transcriptional activation.

Coactivators Potentiate Repression of ERβ485 Wild Type and AF-2 Mutants. Ligand-dependent activation by nuclear receptors is mediated by coactivators, which bind to the AF-2 surface (38, 39). However, it is not known whether these factors participate in ligand-dependent repression. We examined whether GRIP1 enhances repression activity by using only 50 ng of ERβ485, because 1 μg produces a profound repression (80%). The 20% repression of the TNF-RE produced by 50 ng ERβ485 was enhanced twofold (40%) by overexpressing GRIP1 (Fig. 4*B*). Similar results were also observed for ERα (data not shown). Previously, it has been shown that overexpressing GRIP1 restores the loss of ligand-dependent activation by thyroid hormone receptor AF-2 mutants (35, 41).

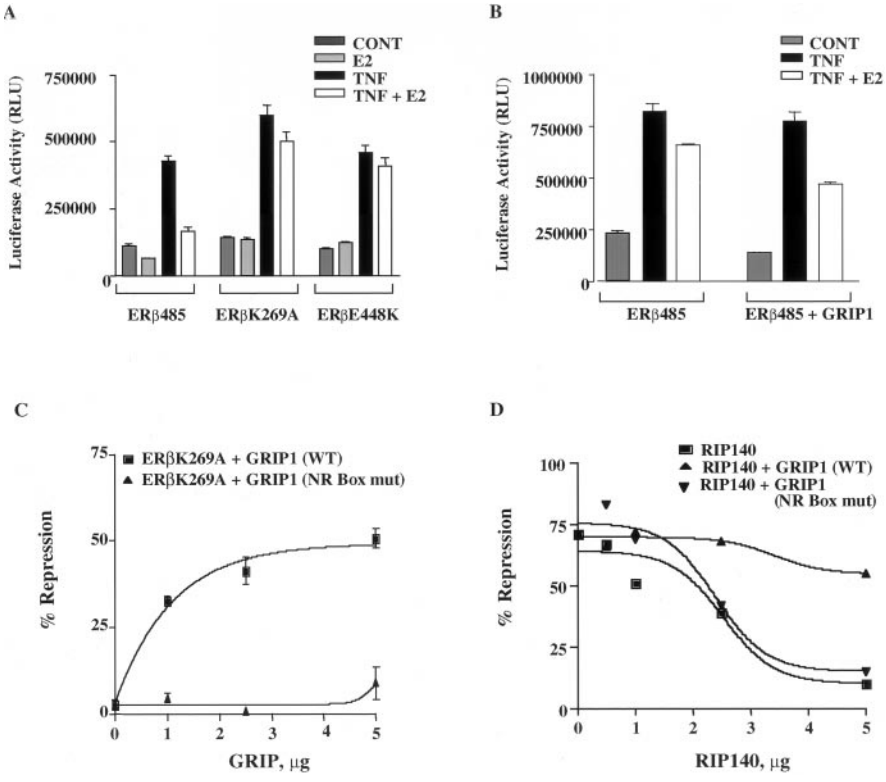

Fig. 4. Mutations in the ERβ485 AF-2 surface impair repression of TNF-RE TKLuc. (*A*) U937 cells were transfected with 3 μg of TNF-RE TKLuc and 1 μg of wild-type ERβ485 or human ERβ485 AF-2 mutants (helix 3, K269A, and helix 12, E448K, based on ERβ485, which corresponds to K314A and E493K based on hERβ530, respectively). Cells were treated for 24 h with TNF-α (5 ng/ml) in the absence or presence of 10 nM E_2 and then assayed for luciferase activity. (*B*) Overexpressing GRIP1 enhances repression activity of ERβ485. Cells were co-transfected with 3 μg of TNF-RE TKLuc and 50 ng of ERβ485 and in the absence or presence of 5 μg of pSG5-GRIP1. All cells were treated for 24 h with TNF-α (5 ng/ml) in the absence or presence of 10 nM E_2. (*C*) Overexpressing GRIP1 restores repression activity of the helix 3 ERβ485 AF-2 mutant (K269A). Cells were transfected with 3 μg of TNF-RE TKLuc and 1 μg of ERβK269A in the presence of increasing amounts of pSG5–wild-type (WT) GRIP1 or pSG5-GRIP1 NR box II and III mutant (mut). All cells were treated for 24 h with TNF-α (5 ng/ml) in the presence of 10 nM E_2. The data are expressed as per cent repression of the TNF-α activation of the TNF-RE. (*D*) RIP140 blocks E_2 repression of the TNF-RE in the presence of wild-type ERβ485. Cells were transfected with 3 μg of TNF-RE TKLuc and 1 μg of ERβ485 in the presence of increasing amounts of an expression vector for RIP140 and 5 μg of pSG5-wild-type (WT) GRIP1 or pSG5-GRIP1 NR box II and III mutant. All cells were treated for 24 h with TNF-α (5 ng/ml) in the presence of 10 nM E_2 and then assayed for luciferase activity.

Thus, we examined whether overexpressing GRIP1 restores repression activity of the ERβ485 AF-2 mutants. Overexpression of GRIP1 restored repression of the helix-3 mutant from 2% to 50% (Fig. 4*C*) and the helix-12 mutant from 0% to 35% in the presence of E_2 (data not shown). Unlike E_2, the anti-estrogens failed to restore repression activity of the AF-2 mutants in the presence of overexpressed GRIP1 (data not shown). Furthermore, a GRIP1 mutant (NR boxes II and III) that is unable to interact with the AF-2 surface of ERβ (42) was ineffective at restoring repression of the ERβ485 AF-2 mutants (Fig. 4*C*). Thus, overexpression of GRIP1 can rescue the loss of ERβ485-mediated repression activity, and this effect requires intact NR boxes that bind to the AF-2 surface.

Repression Is Blocked by RIP140. The results with the anti-estrogens, ERβ485 AF-2 mutants, and GRIP1 overexpression indicate that repression requires direct ER AF-2/coactivator interaction. We hypothesized that factors that lack intrinsic activity that interact with the ER AF-2 surface should block E_2-dependent repression by preventing the binding of endogenous coactivators to the AF-2 surface. To test this hypothesis, cells were transfected with the inactive coactivator RIP140 (data not shown), which exhibits ligand-dependent binding to the ER AF-2 surface (43) and blocks the positive E_2 response (44). Fig. 4*D* shows that RIP140 blocks repression of TNF-RE TKLuc by wild-type ERβ485. The antagonistic action of RIP140 on repression is overcome by overexpressing wild-type GRIP1, but not by the GRIP1 NR box mutant (Fig. 4*D*). These results demonstrate that RIP140 blocks repression in response to E_2, presumably by binding to the transfected ERs through its NR boxes, which may prevent the binding of endogenous coactivators or other coregulators that bind to the AF-2 surface.

Discussion

E_2 inhibits TNF-α production in human peripheral monocytes (20, 21). In these studies, we demonstrate that E_2 represses basal activity and TNF-α induction of the TNF-α promoter. These results suggest that E_2 inhibits TNF-α production by repressing transcription. TNF-α activation and E_2 repression was mapped to the −125 to −82 region of the TNF-α promoter, known as the TNF-RE (23). An AP-1-like site (5′-TGAGCTCA-3′) at −105, which binds c-jun (23) and forms a complex with ATF-2 (31), is essential for TNF-α activation and E_2-mediated repression of the TNF-α promoter, because both activities are abolished when this site is mutated. Repression by E_2 contrasts with our previous studies (33), which showed that the collagenase AP-1 site is activated by E_2 in other cell types. Like the TNF-α promoter, the collagenase promoter is repressed by E_2 in U937 cells, demonstrating that different transcriptional responses to E_2 are cell type specific. However, the finding that E_2 repression of the TNF-α promoter is greater than the collagenase promoter indicates that those elements flanking the AP-1 site in the TNF-α promoter contribute to repression. We speculate that the NF-κB site (32) adjacent to the c-jun site most likely accounts for greater repression of the TNF-α promoter compared with the collagenase promoter, because TNF-α activates NF-κB and ER directly interacts with NF-κB (45) to block its binding to the IL-6 promoter (46). These results suggest that E_2 may repress the TNF-α promoter by disrupting interactions between factors bound to the AP-1-like site and NF-κB, because an NF-κB interaction with the c-jun complex is required for lipopolysaccharide induction of the TNF-α promoter (32).

ERα and ERβ also markedly inhibited TNF-α activation of the TNF-RE upstream of the TK promoter. Surprisingly, ERβ is more potent than ERα at repressing the TNF-RE and the −1044 TNF-α promoter. The different repression activity between ERα and ERβ may be related to differences in the level of receptor expression from transfected plasmids. However, this seems unlikely because the EC_{50} values for activation by ERα and ERβ are similar, whereas the IC_{50} for ERβ is lower than that for ERα for repression. Furthermore, ERα is much more effective than ERβ at activating the ERE in the same cells and conditions. Although *in vivo* data are necessary to confirm that ERβ is more potent than ERα at repression, our results raise the possibility that two ERs exist because ERβ may function more predominantly as a transcriptional repressor. Indeed, selective transcriptional activity by ERα and ERβ may explain some differential tissue-specific and clinical responses to various estrogen analogs.

ERs contain an E_2-dependent AF-2 surface in the LBD that mediates activation of gene transcription by recruiting p160 coactivators (38, 39). Our studies with the ERα deletion mutants and GAL ERα-LBD demonstrate that the E_2-dependent repression function is also located in the LBD. Furthermore, our studies provide evidence that E_2-mediated repression and activation share similarities, which indicates that the AF-2 surface is also required for transcriptional repression. Recently, the anti-estrogens tamoxifen and raloxifene have been shown to prevent the formation of an active AF-2 surface (34, 36). The observation that anti-estrogens antagonize E_2-mediated repression of the TNF-RE suggests that repression is mediated by the AF-2 surface. A role for the AF-2 surface in repression was further demonstrated by the finding that repression is severely impaired with mutations in the AF-2 surface. Our hypothesis that the AF-2 surface mediates repression is also supported by the observation that RIP140 blocks repression by ERβ wild type. It seems likely that RIP140 blocks repression by competing for endogenous coregulatory factors in U937 cells that interact with the AF-2 surface to trigger the repression pathway, because RIP 140 binds to the AF-2 surface and competes for binding of steroid receptor coactivator-1a to the AF-2 surface (43). Thus, like transcriptional activation, repression of the TNF-RE by E_2 is impaired by all known methods that interfere with or block the AF-2 surface, including anti-estrogens, mutations in the AF-2 surface, and RIP140. These results suggest that the AF-2 surface mediates both transcriptional activation and repression activity.

The AF-2 surface of ERs is probably required for repression activity because of its capacity to recruit coregulatory factors. Whereas the coregulatory factors that bind the AF-2 surface to mediate repression of the TNF-α promoter are unknown, the p160 coactivators are potential candidates because they bind to the AF-2 surface of nuclear receptors and mediate positive responses (38, 39). We found that overexpressing GRIP1 enhanced the repression activity of wild-type ERα and ERβ485 and restored the loss of repression activity of ERβ485 AF-2 mutants on the TNF-RE. There are two major interpretations of the results with the ERβ485 AF-2 mutants. First, GRIP1 restores repression activity to the ERβ485 AF-2 mutants by binding to a surface other than the AF-2 surface. This is unlikely because the restoration of repression requires a functional coactivator/AF-2 surface interaction, as demonstrated by the inability of anti-estrogens and the GRIP1 NR box mutant to restore repression activity. Second, mutations in helix 3 and helix 12 do not destroy the AF-2 surface, but decrease the binding affinity for GRIP1. In this case, small amounts receptors prepared in an *in vitro* transcriptional/translational system are not sufficient to overcome the reduced affinity, which can account for the lack of binding of the AF-2 mutants to glutathione *S*-transferase–GRIP1. In contrast, overexpressing GRIP1 in cells may lead to a level that is sufficient to overcome the reduced affinity and allow some GRIP1 binding to the AF-2 surface, even though the surface is mutated. Several observations are consistent with this notion. We found that mutations outside the AF-2 surface had minimal effect on repression. If another surface were responsible for the effects of GRIP1, then mutations outside the AF-2 surface should have impaired repression. Furthermore, cocrystallization of a GRIP1 peptide containing the NR boxes showed that the peptide bound exclusively to the AF-2 surface of ERα (36) and thyroid hormone receptor (37). Finally, our results are consistent with the

observations that overexpressing GRIP1 restores ligand-dependent activation of thyroid hormone receptor AF-2 mutants (35, 41). Thus, we have found that GRIP1 and other p160 coactivators (data not shown) can enhance and restore repression activity, suggesting that some p160s can function as a ligand-dependent coactivator or corepressor of gene transcription. However, it is possible that other coregulatory factors, which have not been identified, also interact with the AF-2 surface to mediate repression.

Whereas the AF-2 surface and coactivators are involved in activation and repression, several features indicate that the mechanism of repression is different from ER activation at an ERE. Unlike activation at a classical ERE, repression does not require the ER DBD. Furthermore, ER does not bind directly to the TNF-RE to trigger repression. The observation that ERβ is more potent than ERα at repression, but less effective than ERα at activating an ERE also suggests that the mechanism of repression is distinct from activation. Finally, we found that, in contrast to repression, overexpressing GRIP1 fails to restore the activation of the same ERα (35) or ERβ AF-2 mutants at an ERE to the same extent (data not shown). The differences in activation and repression by ERs are probably related to distinct ER/coactivator interactions with promoter elements. Most likely, the activation pathway is triggered after the ER/coactivator complex directly binds to an ERE, whereas the repression pathway is probably triggered by protein–protein interactions between the ER/coactivator complex and transcription factors that bind to the TNF-α promoter, such as c-jun, ATF-2, and NF-κB. This model is consistent with the findings that ER and coactivators (steroid receptor coativator-1 and CREB-binding protein) can interact with c-jun, ATF-2, and NF-κB (47–50). Repression of the TNF-RE is also distinct from activation at other AP-1 elements, because raloxifene and tamoxifen act as powerful agonists at these sites (27, 33), whereas they act as antagonists of E_2-mediated repression at the TNF-RE.

Clarifying the molecular mechanisms of estrogen regulation of gene transcription is key to the development of a new generation of more selective estrogens for hormone replacement. Our studies have identified a new role for the AF-2 surface and p160 coactivators in E_2 regulation of transcription, which may prove to be a general feature of ligand-dependent repression by some nuclear receptors. The identification of the molecular mechanisms and coregulatory factors involved in E_2-mediated repression of gene transcription may lead to the development of a safer generation of estrogens for hormone replacement.

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