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Distinct Roles of Unliganded and Liganded Estrogen Receptors in Transcriptional Repression

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Summary

The decline in estrogen levels during menopause is associated with increased cytokine production and inflammatory diseases. Estrogens exert anti-inflammatory effects by repressing cytokine genes, such as *tumor necrosis factor-α* (*TNFα*). The mechanisms involved in transcriptional repression by estrogens are virtually unknown. Here, we used chromatin immunoprecipitation to investigate how estrogens repress the autoinduction of the *TNFα* gene. TNFα assembled a transcriptional activation complex at the TNFα promoter that includes c-*jun*, p50-NFκB, p65-NFκB, CBP, Hsp90, and unliganded estrogen receptor (ER). Estradiol repressed *TNFα* gene expression by reversing the ligand-independent activation by ERα and the stimulatory actions of c-*jun*, NFκB, and CBP on transcription. Silencing of GRIP1 reversed the repression of *TNFα* and other cytokine genes by estradiol, demonstrating that GRIP1 is required for transcriptional repression and can act as a corepressor. Our study demonstrates that ERα is a TNFα-induced coactivator that becomes a repressor in the presence of estradiol by recruiting GRIP1.

Introduction

Estrogens produce biological effects by binding to two distinct estrogen receptors, ERα and ERβ, that can activate or repress gene transcription (Green et al., 1986; Kuiper et al., 1996). Most studies on gene regulation by estrogens have focused on understanding how estradiol (E_2) induces gene transcription (Bjornstrom and Sjoberg, 2005; Nilsson et al., 2001). However, transcriptional repression of cytokine genes may be an important mechanism whereby estrogens prevent inflammatory diseases associated with menopause. As estrogen levels drop after menopause, there is a greater production of cytokines (Pfeilschifter et al., 2002) that contribute to inflammatory conditions, such as osteoporosis (Ammann et al., 1997; Pacifici, 1996), cardiovascular disease (Pai et al., 2004), and Alzheimer's disease (Cacquevel et al., 2004).

Despite the clinical importance of transcriptional repression, the mechanisms of estrogen-mediated repression of cytokine genes are poorly understood. The little that is known has been derived exclusively from transfection studies with promoters linked to reporter genes (An et al., 1999; Galien and Garcia, 1997; Stein and Yang, 1995). These studies suggest that estrogens repress genes by blocking the binding of NFκB to the promoter, but this hypothesis has not been evaluated with endogenous cytokine genes.

As transcriptional activation of proliferative genes by estrogens is associated with breast cancer (Foster et al., 2001) and the transcriptional repression function of ER mediates the anti-inflammatory effects of estrogens, we hypothesize that repression-selective estrogens may retain beneficial effects on bone and other tissues without promoting cancer. Thus, a greater understanding of the molecular mechanisms of transcriptional repression might lead to the discovery of estrogenic drugs that selectively trigger the repression function of ERs. Chromatin immunoprecipitation (ChiP) has been used to study how estrogens activate genes (Metivier et al., 2003; Shang et al., 2000). Here, we used chromatin immunoprecipitation to investigate how estrogens repress transcription of the *TNFα* gene, because excessive TNFα production is associated with osteoporosis (Ammann et al., 1997; Nanes, 2003; Pacifici, 1996) and other inflammatory diseases (Pai et al., 2004).

Results

E_2 Inhibits TNFα Activation of the *TNFα* Gene in Primary Human Osteoblasts and Stable U2OS Cell Lines

We investigated if E_2 represses TNFα activation of the *TNFα* gene in primary human osteoblasts. TNFα produced a large induction of TNFα mRNA, which was markedly inhibited by E_2 (Figure 1A). To generate a sufficient quantity of cells required to investigate the mechanisms whereby estrogens repress the transcription of the *TNFα* gene, we created stable U2OS osteosarcoma cell lines that express a tetracycline-inducible ERα or ERβ (Kian Tee et al., 2004). TNFα produced a large activation of the *TNFα* gene in U2OS-ERα and U2OS-ERβ cells (Figure 1B). E_2 caused a time-dependent repression of TNFα induction of TNFα mRNA levels with both ERα and ERβ. Maximal repression occurred after 3 hr and persisted for at least 18 hr. No repression was observed in U2OS wild-type or MDA-MB-435 cells (data not shown), which do not express ERs, demonstrating that ER mediates the repression by E_2. Imperial Chemical Industries (ICI) 182,780 and tamoxifen did not reduce TNFα mRNA, whereas a small repression was observed with raloxifene (Figure 1C). Other ER isoforms such as ERα46 are expressed in osteoblasts (Denger et al., 2001), which may exhibit different responses to E_2 and SERMs than ERα66 or ERβ1 used in our studies.

To rule out that repression of the *TNFα* gene results from overexpression of ER, doxycycline was used to vary the levels of ERα (Figure 1D). Doxycycline produced a dose-dependent increase in ERα as measured by specific ^{3}H-E_2 binding in intact U2OS-ERα cells (Figure 1E). Repression by E_2 was observed at 0.1 ng/ml

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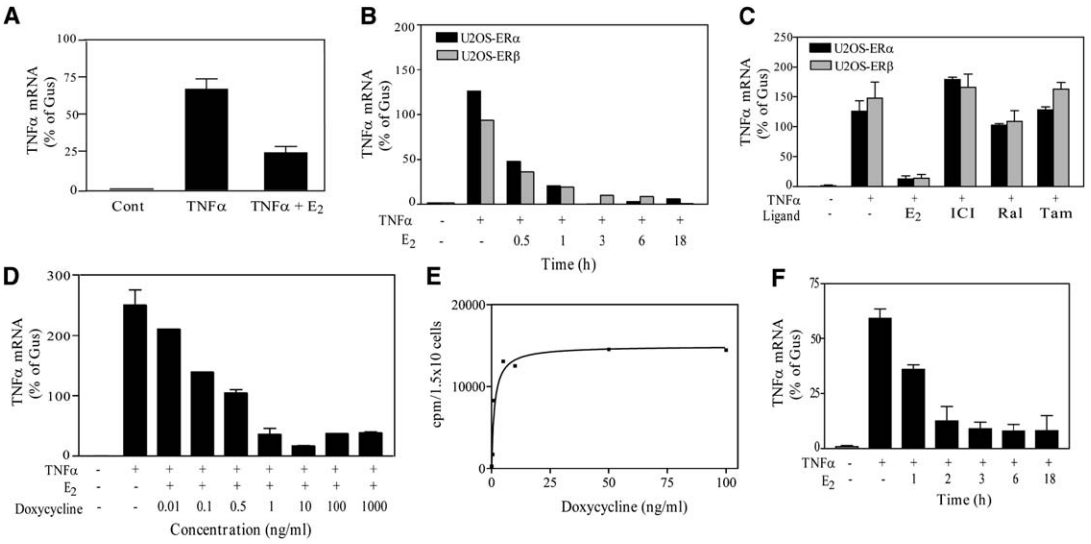

Figure 1. E_2 Represses Transcription of the *TNF*α Gene in Primary Human Osteoblasts and Stable U2OS-ER Cells

(A) Primary human osteoblasts (Clonetics NHOst, Cambrex) were treated with 5 ng/ml TNFα for 2 hr in the absence or presence of 100 nM E_2 for 3 hr. TNFα mRNA levels were determined by qPCR.

(B) U2OS-ERα and U2OS-ERβ cells were treated with 1 μg/ml doxycycline for 18 hr to induce ER expression. Cells were then treated with 5 ng/ml TNFα for 2 hr in the absence or presence of 100 nM E_2 for the indicated times and TNFα mRNA levels were determined by qPCR.

(C) U2OS-ERα and U2OS-ERβ cells were treated for 3 hr with 100 nM E_2, 100 nM ICI, 100 nM raloxifene, or 1 μM tamoxifen and with 5 ng/ml TNF for 2 hr. TNFα mRNA levels were determined by qPCR.

(D) U2OS-ERα cells were treated with doxycycline for 18 hr. After treatment with E_2 and TNFα, qPCR was used to measure TNFα mRNA.

(E) ER binding assays were done for 2 hr at 37ºC with ^{3}H-E_2 in U2OS-ERα cells treated with doxycycline.

(F) U2OS-Flag-tagged-ERα cells were treated with TNFα and E_2 as described above for U2OS-ERα cells. TNFα mRNA levels were determined by qPCR. Error bars represent the mean ±SEM.

doxycycline (Figure 1D), which produced 825 receptors per cell (Figure 1E). At 0.5 ng/ml doxycycline, E_2 produced a 60% reduction in TNFα mRNA in U2OS-ERα cells that contained 4800 receptors per cell. These levels are comparable to 1500–3300 receptors per cell expressed in primary human osteoblasts (Eriksen et al., 1988) and less than the 15,000 receptors per cell expressed in MCF-7 cells (McLaughlin et al., 1984), which are commonly used to study how ERs activate gene transcription (Metivier et al., 2003; Shang et al., 2000). These studies demonstrate that inducible-U2OS cell lines behave similarly to primary human osteoblasts and that repression of the *TNF*α gene is observed at physiological levels of ERs.

TNFα Recruits c-*jun*, NFκB, and Unliganded ER to the TNFα Promoter

ChIP was used to identify factors that TNFα recruits to the TNFα promoter. Polymerase chain reaction (PCR) primers used spanned the TNFα-response element (TNF-RE; −125 to −82 region), which contains a composite AP-1/NFκB element (Tzagarakis-Foster et al., 2002) that mediates TNFα activation and E_2 repression (An et al., 1999). U2OS cells were stably transfected with Flag-ERα or Flag-ERβ to initially compare the binding of ERα and ERβ with a single antibody. E_2 repressed *TNF*α gene expression in Flag-ERα (Figure 1F) and Flag-ERβ (data not shown) cells equivalent to the inducible cells (Figure 1B), demonstrating that Flag-ERs behave similarly to nontagged ERs. Cells were treated for increasing times with TNFα in the absence or presence of E_2, and then ChIP was done by using antibodies to c-*jun*, p50-NFκB, p65-NFκB, and the Flag epitope tag. TNFα induced recruitment of c-*jun*, p50-NFκB, and p65-NFκB to the TNF-RE in U2OS-Flag-ERα and U2OS-Flag-ERβ cells (Figures 2A and 2B, respectively). Unexpectedly, TNFα also recruited ERα and ERβ to the *TNF*α gene in the absence of E_2 (Figures 2A and 2B, respectively). Maximal recruitment of unliganded ER occurred after 3 to 4 hr of TNFα treatment, which was slower than the recruitment of c-*jun* and NFκB. E_2 did not alter the recruitment of c-*jun*, p50-NFκB, or p65-NFκB, but resulted in the apparent loss of Flag-ERα and Flag-ERβ from the TNFα promoter. The disappearance of ER was not due to an unusual characteristic of the Flag-ER fusion proteins or Flag antibody, because the loss was observed with three antibodies against different epitopes of ERα and antibodies against ERβ (Figure 2C). Furthermore, E_2 recruited ERα and ERβ to the *keratin 19* gene (Figures 2C), which is activated by E_2 in U2OS-ERα and U2OS-ERβ cells (Figure 2D). TNFα did not recruit unliganded ERα or ERβ to the *keratin 19* gene at any time during a 4 hr time course (Figure 2E). These results demonstrate that TNFα recruits ER to the *TNF*α gene, but not to the *keratin 19* gene, whereas E_2 recruited ER only to the *keratin 19* gene.

Unliganded ERα Is Associated with c-*jun* and NFκB

To explore the mechanism by which TNFα recruits unliganded ER to the TNFα promoter, we performed coimmunoprecipitation to investigate protein-protein interactions with ERα. U2OS-ERα cells were treated with TNFα in the absence or presence of doxycycline to induce ERα. Cell extracts were immunoprecipitated with

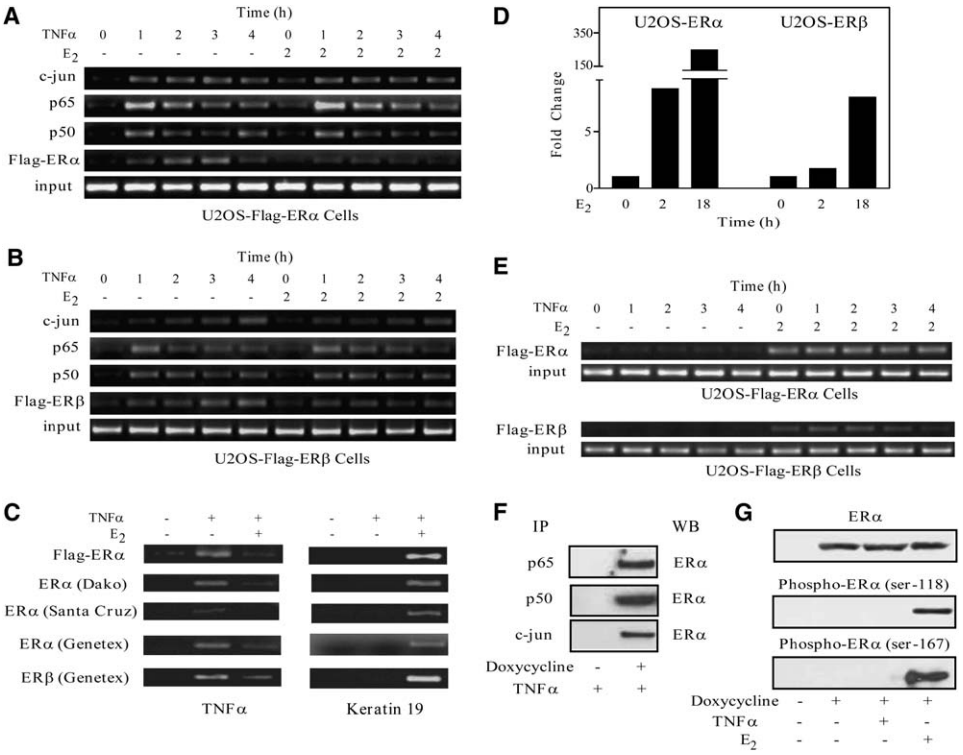

Figure 2. TNFα Recruits c-*jun*, NFκB, and Unliganded ERα to the *TNF*α Gene

(A) U2OS-Flag-ERα cells were treated with 5 ng/ml TNFα for increasing times in the presence or absence of E_2. ChIP was performed with antibodies to p50-NFκB, p65-NFκB, c-*jun*, or Flag epitope tag. Immunoprecipitated chromatin fragments were amplified by PCR, and products were visualized with ethidium bromide.

(B) ChIP assays in U2OS-Flag-ERβ cells were done as described for U2OS-Flag-ERα cells.

(C) ChIP in U2OS-ERα and U2OS-ERβ cells was done by using the indicated ER antibodies. DAKO and GeneTex antibodies recognize an epitope in the N terminus, whereas the Santa Cruz antibody recognizes an epitope in the C terminus.

(D) U2OS-ERα and U2OS-ERβ cells were treated with E_2 for the indicated times. Keratin 19 mRNA levels were determined by qPCR.

(E) U2OS-Flag-ERα and U2OS-Flag-ERβ cells were treated as indicated, and immunoprecipitated chromatin fragments were then PCR amplified by using primers that spanned the keratin 19 ERE.

(F) U2OS-ERα cells were treated with doxycycline (lane 2) and TNFα. Cell lysates were immunoprecipitated (IP) with antibodies to p50-NFκB, p65-NFκB, or c-*jun*. Immunoprecipitated proteins were detected by Western blots (WB) with an ERα antibody.

(G) U2OS-ERα cells were treated with doxycycline, TNFα, or E_2 as indicated. Cell lysates were run on SDS-PAGE. Immunoblotting was performed with antibodies to ERα (upper panel), phospho-ERα (Ser-118), or phospho-ERα (Ser-167).

antibodies to c-*jun*, p50-NFκB, or p65-NFκB. Western blotting analyses were done with an ERα antibody. In cells treated with TNFα and doxycycline, c-*jun* and NFκB coimmunoprecipitated with ERα (Figure 2F). No signal was observed with ERα antibodies in control cells that did not express ERα (-doxycycline). These results suggest that c-*jun* and NFκB form a platform to tether ER to the TNFα promoter.

Membrane receptor signaling can activate the mitogen-activated protein kinase (MAPK) pathway, which causes phosphorylation of serine (ser)-118 and ser-167 in the activation function-1 (AF-1) domain of ERα (Lannigan, 2003). TNFα activates MAPK (Wajant et al., 2003), suggesting that TNFα may trigger the interaction of ER with c-*jun* and NFκB by inducing ER phosphorylation. To examine this possibility, U2OS-ERα cells were treated with TNFα, and Western blots were performed with phospho-ERα antibodies that recognize ser-118 or ser-167. E_2, which is known to activate MAPK (Improta-Brears et al., 1999; Kato et al., 1995; Le Goff et al., 1994), phosphorylated both ser-118 and ser-167 (Figure 2G, middle and lower panels, respectively). In contrast, TNFα did not phosphorylate ser-118 and ser-167, even though ERα was present in all samples except for cells that were not treated with doxycycline (Figure 2G, upper panel). These results demonstrate that TNFα does not activate ER by phosphorylating these two serines.

Unliganded ER Acts as a Coactivator by Potentiating TNFα-Induced Transcription of the *TNF*α Gene

The significance of unliganded ER interaction with the *TNF*α gene was studied in U2OS-ERα cells. The induction of ERα by doxycycline was associated with a much greater TNFα stimulation of TNFα mRNA (Figure 3A). The potentiation required ER, because no potentiation of TNFα activation was observed in wild-type U2OS cells treated with doxycycline (Figure 3A). To rule out that unliganded ER potentiation is not due to the selection of an anomalous stable cell line, an adenovirus (Ad) was used to introduce ERα into wild-type U2OS cells, which lack ERα (Figure 3B insert, lane −). Cells infected with Ad-ERα produced ERα (Figure 3B insert, lane +) and exhibited a greater induction of TNFα

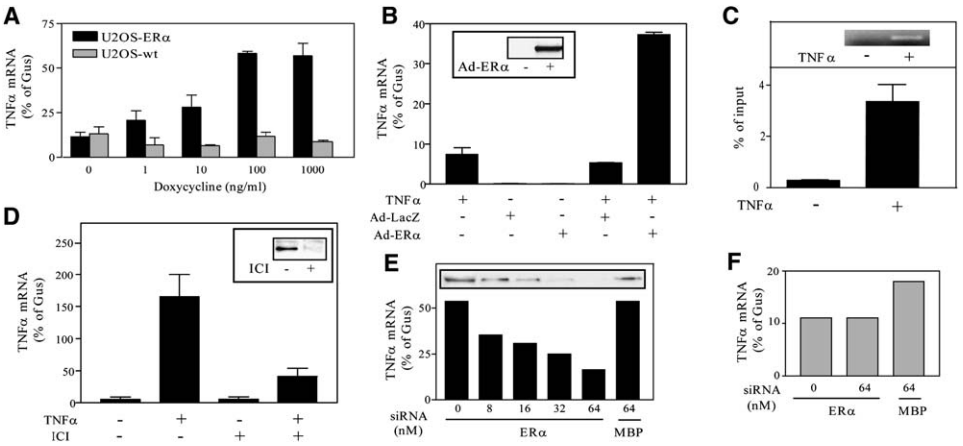

Figure 3. Unliganded ERα Potentiates TNFα Activation of the *TNFα* Gene

(A) U2OS-ERα or wild-type (wt) U2OS cells were treated for 18 hr with the indicated concentration of doxycycline. TNFα (0.1 ng/ml) was added for 3 hr, and qPCR was used to measure TNFα mRNA levels.

(B) Wt U2OS cells were infected for 24 hr with 50 MOI Ad-ERα or Ad-LacZ and then treated with 0.25 ng/ml TNFα for 2 hr. TNFα mRNA was determined by qPCR. Immunoblotting of cell extracts from cells infected with Ad-LacZ (−) or Ad-ERα (+) was done with an ERα antibody (insert).

(C) ChIP was done in MCF-7 cells in the absence or presence of TNFα (3 hr treatment) by using an ERα antibody. PCR products were visualized with ethidium bromide (insert) or were measured by qPCR (bars).

(D) MCF-7 cells were treated with ICI for 24 hr. TNFα was added for 2 hr, and TNFα mRNA was determined by qPCR. ERα protein expression was assessed by immunoblotting (insert).

(E) ERα or MBP siRNA were transfected at the amount indicated into MCF-7 or (F) MDA-MB-435 cells for 4 days. Cells were treated with TNFα for 2 hr. TNFα mRNA was measured by qPCR. Immunoblots were performed by using cell extracts from siRNA transfected cells. Relative levels of ERα were detected with an ERα antibody. Error bars represent the mean ±SEM.

mRNA compared to uninfected cells or cells infected with the control virus Ad-LacZ (Figure 3B, bars).

To determine if unliganded ER potentiates TNFα-induction of the *TNFα* gene in a cell type that naturally expresses ERα, we studied MCF-7 cells. TNFα induced recruitment of ERα to the *TNFα* gene as demonstrated by the images of PCR products spanning the TNF-RE (Figure 3C, insert, lane +) and confirmed by real-time quantitative PCR (qPCR) (Figure 3C, bars). To explore if ERα acts as a coactivator in MCF-7 cells, we treated cells with ICI, which causes ER degradation (Fan et al., 2003). ICI produced a marked reduction in ERα levels as determined by immunoblotting (Figure 3D, insert, lane +), which coincided with a lower induction of TNFα mRNA compared to untreated MCF-7 cells (Figure 3D, bars). A similar reduction in TNFα activation of the *TNFα* gene occurred in MCF-7 cells expressing lower levels of ERα (Figure 3E, insert) after treatment with short interfering RNA (siRNA) to ERα (Figure 3E, bars). No decline in TNFα activation of TNFα mRNA occurred with control siRNA to maltose binding protein (MBP). Furthermore, siRNA to ERα did not inhibit TNFα activation of the *TNFα* gene in ER negative MDA-MB-435 cells (Figure 3F). This data provide further evidence that unliganded ERα acts as a coactivator by potentiating the stimulatory effects of TNFα.

Unliganded ER Does Not Potentiate TNFα-Induced Transcription of the *TNFα* Gene by Recruiting p160 Coactivators or CBP

Membrane receptors signaling pathways activate unliganded nuclear receptors (Power et al., 1991), which can stimulate transcription by recruiting p160 coactivators to AF-1 of ER (Dutertre and Smith, 2003; Webb et al., 1998). To investigate if the coactivator activity of ERα is due to the recruitment of p160s, Flag-ERα cells were treated with TNFα for 3 hr and then ChIP was performed by using antibodies to Flag-ERα or p160s (Anzick et al., 1997; Hong et al., 1996; Smith and O'Malley, 2004; Voegel et al., 1996). As previously shown, TNFα recruited ERα, but not the p160s, SRC-1, GRIP1 (TIF2/NcoA2/SRC-2), or AIB1 (SRC-3) (Figure 4A, lane 2). These results demonstrate that ERα does not enhance TNFα-induced transcription of the *TNFα* gene by recruiting these coactivators. Another possibility is that ERα activates the *TNFα* gene by recruiting CBP (Kamei et al., 1996). ChIP shows that TNFα recruited CBP in U2OS-ERα cells (Figure 4A, lane 2), but recruitment was also observed in cells not induced with doxycycline to produce ERα (Figure 4B, lane +). These studies provide evidence that the coactivation activity of unliganded ER is not likely due to the recruitment of p160s or CBP.

TNFα Assembles a Complex at the Promoter that Includes Unliganded ERα, Hsp90, and CBP but not GRIP1

In addition to ERα and CBP, TNFα recruited heat shock protein 90 (Hsp90), but not GRIP1, to the TNFα gene (Figure 4A, lanes 2). We used re-ChIP to determine if these factors are recruited simultaneously to the TNFα promoter. Similar to ChIP data from the Flag-ERα cells, re-ChIP data show that TNFα recruited unliganded ERα and Hsp90 simultaneously to the TNFα promoter in the U2OS-ERα cells (Figure 4C). These results provide additional evidence that unliganded ERα is recruited to the TNFα promoter, because Hsp90 is known to interact with unliganded nuclear receptors (Pratt, 1997). TNFα also induced the simultaneous recruitment of ERα and CBP (Figure 4D), but not CBP and GRIP1 (Figures 4E). The combined data from ChIP and re-ChIP studies

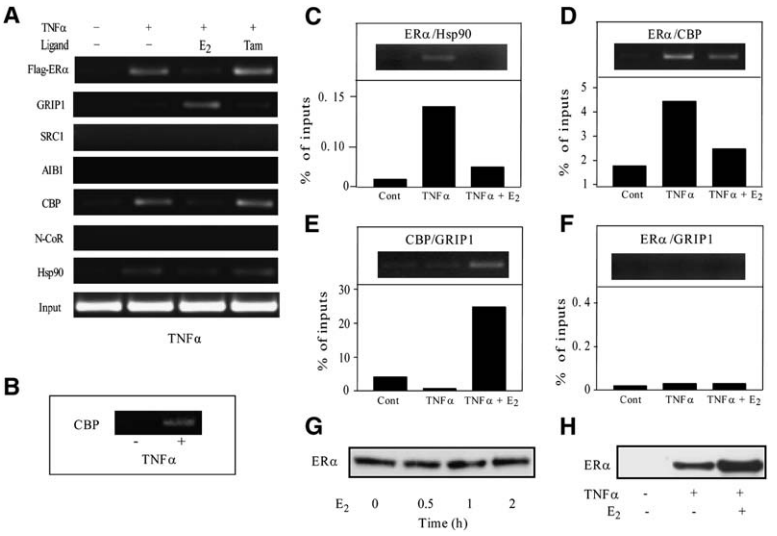

Figure 4. TNFα Recruits Unliganded ERα, Hsp90, and CBP to the *TNFα* Gene

(A) U2OS-Flag-ERα cells were treated with 5 ng/ml TNFα for 3 hr followed by 100 nM E_2 or 1 μM tamoxifen for 2 hr. ChIP was performed with antibodies to Flag-ERα, GRIP1, SRC-1, AIB1, CBP, NCoR, or Hsp90. Immunoprecipitated chromatin fragments were amplified by PCR, and the products were visualized with ethidium bromide.

(B) U2OS-ERα cells not induced with doxycycline were untreated (−) or treated with (+) TNFα for 2 hr. ChIP was done by using an antibody to CBP. DNA fragments were amplified by PCR with primers for the TNF-RE.

(C–F) For re-ChIP studies, cells were treated with 5 ng/ml TNFα for 3 hr followed by 100 nM E_2 for 2 hr and subjected to ChIP. After primary immunoprecipitation, crosslinked complexes were eluted and reimmunoprecipitated with a second antibody. The two antibodies used are indicated in the figures. The reimmunoprecipitated material was used for semiquantitative PCR (insert) and qPCR (bars).

(G) U2OS-ERα cells were treated with doxycycline for 18 hr followed by 100 nM E_2 for the indicated time. ERα was detected by immunoblotting with an ERα antibody.

(H) U2OS-ERα cells were induced with doxycycline and then treated with TNFα and E_2 as indicated. Nuclear extracts were incubated with a biotinylated oligonucleotide containing the TNF-RE attached to streptavidin-coated magnetic beads. The eluted proteins were separated by SDS-PAGE, and immunoblotting was done with an ERα antibody.

demonstrate that TNFα assembles a complex at the TNFα promoter that includes c-*jun*, p50-NFκB, p65-NFκB, Hsp90, CBP, and unliganded ERα, which are likely responsible for the marked induction of the TNFα gene by TNFα.

E_2 Recruits GRIP1 to the TNFα Promoter

As E_2 did not alter recruitment of c-*jun* or NFκB by TNFα (Figure 2), it is unlikely that E_2 causes repression by interfering with binding of these factors to the TNFα promoter. To identify factors responsible for E_2-mediated repression, U2OS-ERα cells were treated with TNFα in the absence or presence of E_2. The addition of E_2 led to the recruitment of GRIP1, but not SRC-1, AIB1, or the corepressor, NCoR (Figure 4A, lane 3). Re-ChIP shows that in the presence of E_2, GRIP1 and CBP are simultaneously present at the TNFα promoter (Figure 4E), demonstrating that E_2 recruits GRIP1 to the transcriptional activation complex created by TNFα. Consistent with our ChIP data showing the loss of ERα with recruitment of GRIP1, re-ChIP indicates that ERα and GRIP1 are not present together at the promoter (Figure 4F).

The apparent loss of ERα after E_2 treatment from the promoter by ChIP (Figures 2 and 4) could result from receptor degradation, the dismissal of ERα from the promoter, or masking of epitopes for ERα antibodies after GRIP1 is recruited. Immunoblotting shows that ERα levels were stable during E_2 treatment (Figure 4G), demonstrating that the loss of ERα is not due to enhanced ERα degradation. To explore if ERα is masked after E_2 treatment, DNA pull-down assays were performed by using a biotinylated double-stranded oligonucleotide containing the TNF-RE. Immunoblots of proteins captured by the TNF-RE show that ERα is present in nuclear extracts prepared from cells treated with TNFα alone or TNFα and E_2, but not untreated cells (Figure 4H). These results suggest that despite the disappearance of ERα by ChIP, it is possible that ERα remains at the promoter with E_2 but is masked by GRIP1 during ChIP.

GRIP1 Acts as a Corepressor of E_2-Mediated Repression of the *TNFα* Gene

Our data showing that tamoxifen did not cause repression of the *TNFα* gene (Figure 1C) or recruit GRIP1 (Figure 4A, lane 4) to the promoter suggest that GRIP1 is required for repression by E_2. A requirement for GRIP1 in repression is supported by our findings with U2OS cells stably transfected with Flag-ERα containing a mutation in helix 3 (K362A) of AF-2, which is known to impair binding of p160s (Feng et al., 1998; Henttu et al., 1997). E_2 did not repress the *TNFα* gene in Flag-ERα-K362A cells (Figure 5A). ChIP shows that TNFα recruited unliganded ERα-K362A to the *TNFα* gene, but E_2 did not recruit GRIP1 (Figure 5B). These results demonstrate that repression by E_2 only occurs when GRIP1 is recruited. To further explore a functional role for GRIP1 in a setting where the AF-2 surface of ERα is not destroyed, we used RNA interference (RNAi) to silence GRIP1. Two lentiviruses expressing short hairpin RNA (shRNA) directed to different regions of the GRIP1 were infected sequentially in U2OS-ERα cells prior to selection with puromycin. No differences in TNFα activation of the *TNFα* gene was observed in cells infected with scrambled or GRIP1 shRNA compared to uninfected U2OS-ERα cells (data not shown). Furthermore, the magnitude of repression by E_2 in the cells expressing the scrambled shRNA was the same as the uninfected U2OS-ERα cells. These results demonstrate that the shRNA did not exhibit any nonspecific effects. Western blot of proteins from U2OS-ERα cells expressing shRNA to GRIP1 showed a 76% knockdown of GRIP1 by quantitative chemiluminescence compared to cells

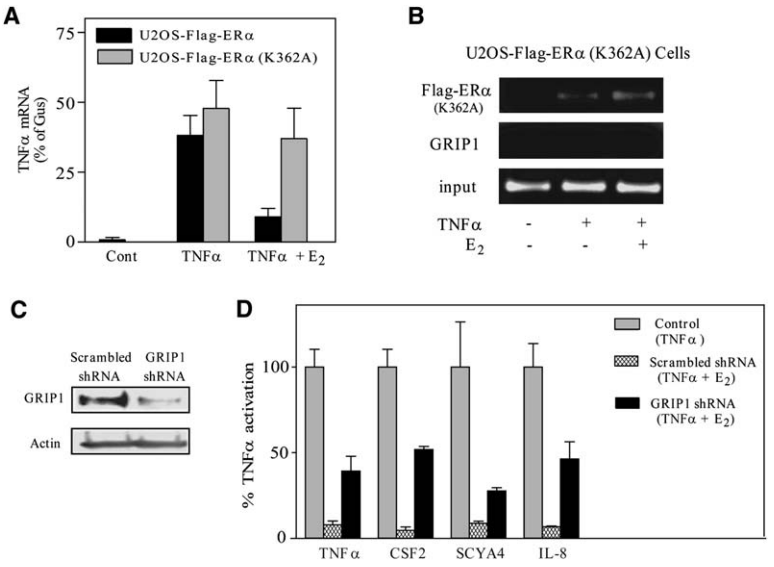

Figure 5. E_2 Recruits GRIP1 to the *TNFα* Gene, which Mediates Repression

(A) U2OS-Flag-ERα and U2OS-Flag-ERα-K362A cells were treated with TNFα in the presence or absence of E_2. TNFα mRNA levels were determined by qPCR.

(B) ChIP for Flag-ERα and GRIP1 was performed in U2OS-Flag-ERα-K362A cells.

(C) Western blot for GRIP1 from cell lysates from U2OS-ERα cells stably transfected with a lentivirus expressing a scrambled or two different GRIP1 shRNA.

(D) U2OS-ERα cells expressing a scrambled or GRIP1 shRNA were treated with 5 ng/ml TNFα for 2 hr in the absence or presence of 100 nM E_2 for 3 hr. TNFα, CSF2, SCYA4, and IL-8 mRNA levels were determined by qPCR. The data is presented as a percentage of TNFα activation (control) in the scrambled or GRIP1 shRNA U2OS-ERα cells. Error bars represent the mean ±SEM from three independent experiments.

expressing a scrambled shRNA (Figure 5C). E_2 inhibited the TNFα activation of the *TNFα* gene by 90% in U2OS-ERα cells expressing scrambled shRNA (Figure 5D). In cells expressing shRNA to GRIP1, only a 45% inhibition was observed. Silencing of GRIP1 also caused a partial reversal of E_2 repression of other cytokine genes activated by TNFα, including *colony stimulating factor-2* (*CSF2*), *small inducible cytokine A4* (*SCYA4*), and *interleukin-8* (*IL-8*) (Figure 5D). The incomplete reversal of repression by E_2 is most likely due to the presence of residual GRIP1 protein (Figure 5C). These results demonstrate that silencing of GRIP1 results in a reduction of E_2-mediated repression of TNFα activation of cytokine genes.

Discussion

Understanding how estrogens repress cytokine genes is important because cytokines have been implicated in a variety of inflammatory diseases that increase after menopause (Pfeilschifter et al., 2002). To address the paucity of information on the mechanism of repression of cytokine genes by estrogens, we used ChIP to identify the factors involved in repressing the *TNFα* gene.

TNFα Recruits Unliganded ER to a Platform Made of c-*jun* and NFκB

Our ChIP data demonstrated that TNFα recruits the transcription factors c-*jun*, p50-NFκB, p65-NFκB, and CBP to the promoter, which participates in the induction of the *TNFα* gene. Unexpectedly, TNFα also recruited unliganded ER along with Hsp90 to the TNFα promoter. Pull-down assays (Stein and Yang, 1995), our previous studies showing that mutations in the c-*jun* and NFκB sites abolish E_2 repression of the TNFα promoter (Tzagarakis-Foster et al., 2002), and coimmunoprecipitation data from this study indicate that ER interacts with a platform made of c-*jun* and NFκB. A requirement for the formation of this platform prior to ER binding is supported by our kinetic ChIP data that shows ER is recruited to the *TNFα* gene slower than c-*jun*, p50-NFκB, and p65-NFκB. These studies suggest that a major role of c-*jun* and NFκB is to tether unliganded ER to the TNFα promoter.

The mechanism whereby TNFα induces protein-protein interactions between ER, c-*jun*, and NFκB is unclear. Unliganded nuclear receptors are activated by signal transduction pathways (Power et al., 1991) such as MAPK that phosphorylates ER (Improta-Brears et al., 1999; Kato et al., 1995; Le Goff et al., 1994). TNFα activates MAPK (Wajant et al., 2003), but it did not induce phosphorylation of ser-118 or ser-167. These results suggest that other signal transduction pathways activate ERα in U2OS cells. In contrast to the *TNFα* gene, TNFα did not recruit unliganded ER to the *keratin 19* gene, which is activated by E_2. These findings suggest that in U2OS cells, TNFα selectively recruits unliganded ER to genes repressed by E_2, but not to genes activated by E_2.

Unliganded ER Acts as a Coactivator at the TNFα Promoter

Once TNFα recruits unliganded ERα to the promoter, we demonstrated that ER acts as a coactivator. We showed that ERα produced a marked enhancement of the TNFα-mediated increase in TNFα mRNA in inducible-U2OS cells that expressed increasing levels of ERα with doxycycline or in wild-type U2OS cells infected with an adenovirus that expresses ERα. Furthermore, a loss of ERα in MCF-7 cells by ICI or siRNA resulted in a diminished TNFα induction of the *TNFα* gene. To our knowledge, the coactivator property of unliganded ER at promoters has not been reported for genes repressed by estrogens. Thus, our studies demonstrate that ERα acts as a TNFα-activated coactivator and reveal a unique transcriptional activity for ER.

The N-terminal AF-1 domain of ER is capable of recruiting p160 coactivators (Dutertre and Smith, 2003; Webb et al., 1998). By contrast, no evidence was found in our ChIP studies for TNFα recruitment of p160s to unliganded ER. Furthermore, re-ChIP data showed that ER and GRIP1 were not at the TNFα promoter simultaneously, demonstrating that unliganded ER has a conformation that is unable to interact with p160

coactivators when tethered to the TNFα promoter. We also found that TNFα recruits CBP in the absence of ER. These results demonstrate that the coactivator activity of ER is not likely due to the recruitment of p160s or CBP. Alternatively, ER may enhance the activation of the *TNFα* gene through an intrinsic coactivator mechanism, the recruitment of other p160 coactivators not tested, or by enhancing the activation function of CBP, c-*jun*, NFκB, or other factors bound to the TNFα promoter.

E_2 Initiates Repression by Recruiting GRIP1, which Acts as a Corepressor

Transfection studies suggested that E_2 inhibits the activation of cytokine genes by interfering with the binding of NFκB (Galien and Garcia, 1997; Stein and Yang, 1995). However, this is an unlikely mechanism whereby E_2 represses the *TNFα* gene, because ChIP showed that E_2 did not impair the binding of p55-NFκB or p65-NFκB. Another possible mechanism is that E_2 causes repression by recruiting corepressor proteins such as NCoR, which mediates transcriptional repression by unliganded nuclear receptors (Chen et al., 1996; Horlein et al., 1995) and ER and progesterone receptor antagonists (Zhang et al., 1998). NCoR is also required for the repression of inflammatory genes by PPAR-γ agonists in macrophages (Pascual et al., 2005). However, our studies suggest that NCoR is not involved in estrogen-mediated repression of cytokine genes. We previously found that overexpressing NCoR did not potentiate the repression of the TNFα promoter by E_2 in transfection studies (An et al., 1999). A lack of a role for NCoR in repression is consistent with the finding that E_2 did not recruit NCoR to the *TNFα* gene. The findings with PPAR-γ (Pascual et al., 2005) and ours with ER indicate that agonist-mediated repression by nuclear receptors can occur by distinct mechanisms.

Our studies provide evidence that E_2 triggers repression by binding to ER already present at the promoter. Once ER is liganded with E_2, it recruits GRIP1, which mediates repression of the *TNFα* gene. A role for GRIP1 in E_2-mediated repression was supported by the observation that no recruitment of GRIP1 occurred with tamoxifen, which is known to block p160 interactions with ER (Brzozowski et al., 1997; Shiau et al., 1998). Also, a mutation in helix 3 of ER that disrupts the AF-2 surface (Feng et al., 1998; Henttu et al., 1997) prevented the recruitment of GRIP1. In both cases, repression did not occur when there was no recruitment of GRIP1. Because it is possible that ER must be in an active conformation to induce repression, we also used RNAi to explore the role of GRIP1 in repression. Silencing of GRIP1 reversed the repression of *TNFα* and other cytokine genes by E_2. The series of studies with tamoxifen, the helix 3 mutant, and RNAi demonstrate that GRIP1 functions as a corepressor of cytokine genes repressed by estrogens.

GRIP1 May Be a Unique p160 Protein that Can Act as a Coactivator or Corepressor

Other members of the p160s, including SRC-1 (Onate et al., 1995) and AIB1 (Anzick et al., 1997), were not recruited to the *TNFα* gene. These findings suggest that GRIP1 is selectively recruited to genes repressed by E_2 and that GRIP1 can function as a coactivator or corepressor, whereas SRC-1 and AIB1 function only as coactivators in U2OS cells. This notion is supported by our RNAi data that suggest other coactivators cannot substitute for GRIP1, because if GRIP1 was redundant then no reversal of repression should have occurred in cells depleted of GRIP1 by RNAi. The finding that GRIP1 has a markedly different amino acid composition compared to SRC-1 and AIB1 in a region linked to glucocorticoid receptor (GR) repression of gene transcription also suggests that p160s possess distinct activities (Rogatsky et al., 2002).

Two activation domains (AD1 and AD2) are present in the C-terminal domain of GRIP1 (Hong et al., 1996; Voegel et al., 1996) and activate target genes by recruiting acetyltransferases (Kamei et al., 1996; Ogryzko et al., 1996) and methyl-transferases (Chen et al., 1999). Deletion of both AD1 and AD2 does not affect ER (data not shown) or GR-mediated repression in transfection studies (Rogatsky et al., 2002), indicating that the region of GRIP1 involved in repression is distinct from activation. These findings suggest that GRIP1 may be a unique p160 protein that acts as a corepressor in genes repressed by some steroid receptors.

ER Disappears from the TNFα Promoter with E_2 Treatment

Our ChIP data showed that E_2-mediated recruitment of GRIP1 is associated with the loss of ER from the *TNFα* gene. By contrast, E_2 induced recruitment of both ER and GRIP1 to the *keratin 19* gene, which is consistent with data derived from other genes activated by ER (Metivier et al., 2003; Shang et al., 2000). We showed that the loss of ER was not due to receptor degradation. Another possible explanation is that the epitopes recognized by the ER antibodies were masked when GRIP1 interacts with the ER. This is consistent with the observation that the ERα helix 3 mutant does not disappear from the TNFα promoter when GRIP1 is not recruited. Our DNA pull-down assays with the TNF-RE linked to streptavidin-coated magnetic beads showed that nuclear extracts derived from cells treated with TNFα alone or TNFα and E_2 contained ERα that bound to the TNF-RE. These findings support the data with the helix 3 mutant, indicating that ER remains at the promoter with E_2 treatment but that the epitopes for ER antibodies are being masked during ChIP.

Whereas our studies were unable to determine the mechanism for the disappearance of ERα from the promoter, the observation that ER disappears when using the flag antibody and three antibodies against different epitopes to ERα in the amino- or carboxy-terminal domain and also antibodies to ERβ favors the view that ER is dismissed from the promoter. If ER were actually dismissed, then GRIP1 would have to be tethered to a factor other than ER once a required surface is cleared of ER. The dismissal of ER from the TNFα promoter would abolish the coactivator activity of ER and could also serve the purpose of making ER available to other target genes.

Potential Mechanisms whereby GRIP1 Acts as a Corepressor

The mechanism whereby GRIP1 acts as a corepressor at the *TNFα* gene is not known. It is possible that

GRIP1 interferes with the coactivator activity of unliganded ER, the activation function of transcription factors, such as c-*jun*, NFκB, or CBP, or factors bound to the TNFα promoter involved in transcriptional regulation. Our findings showing that E_2 causes opposite effects on the recruitment of ER to the *TNFα* and *keratin 19* genes indicate that the conformation of GRIP1 is different when it acts as a corepressor instead of a coactivator. When GRIP1 is bound to genes activated by E_2, its conformation exposes surfaces that facilitate recruitment of activators such as histone acetyltransferases and methyl-transferases (Metivier et al., 2003; Shang et al., 2000). In contrast, our studies suggest that when GRIP1 is associated with the TNFα promoter, different surfaces are exposed that might recruit repressor proteins that are actually responsible for repression of the *TNFα* gene.

Clinical Implications of Unliganded and Liganded ER Regulation of the *TNFα* Gene

Whereas it is known that the decline of estrogens during menopause can lead to osteoporosis and other diseases associated with menopause, our findings raise the unexpected possibility that unliganded ERs contribute to some menopausal conditions. The lack of estrogens in postmenopausal women could lead to unopposed coactivator activity of ERs that persist in tissues in response to TNFα. Based on our studies, this would enhance the induction of the *TNFα* gene and increase TNFα production in tissues and thereby promote inflammation and tissue injury. In contrast, prior to menopause, higher levels of estrogens will result in the formation of liganded ERs that recruit GRIP1, which acts a corepressor by reversing the coactivator activity of ER and the stimulatory effects of the c-*jun*, NFκB, and CBP on the transcription of inflammatory genes, such as *TNFα*. By understanding the mechanisms whereby estrogens repress cytokine genes, it may be possible to develop repression-selective estrogens that retain anti-inflammatory properties, but do not activate genes known to promote cancer.

Experimental Procedures

Preparation of Stable Lines

U2OS cells stably expressing tetracycline-inducible ERα or ERβ were prepared as previously described (Kian Tee et al., 2004). Flag-tagged cells were made by transfecting wild-type U2OS cells with pcDNA 6/V5-His (Invitrogen) containing cDNAs for Flag-ERα, Flag-ERβ, or Flag-ERα helix 3 mutant (K362A). The Flag-tag was inserted at the N terminus of ERs. Stable Flag-tagged cell lines were selected with 10 μg/ml blasticidin (Invitrogen).

Quantitative Real-Time PCR

Total RNA was prepared by using TRIzol (Invitrogen) as previously described (Paruthiyil et al., 2004). qPCR detection of TNFα mRNA was performed with a BioRad iCycler Thermal Cycler System with a TNFα TaqMan assay kit (Applied Biosystems). Control reactions were performed by using primers and a probe to detect β-glucoronidase (Gus). Primers for Gus were: forward, 5′-CTCATTTGGAATTTTGCCGATT-3′; reverse, 5′-CCGAGTGAAGATCCCCTTTTTA-3′ (Operon). qPCR detection of *keratin19, CSF2, SCYA4*, and *IL-8* mRNA was performed with the iQ SYBR Green Supermix (Bio-Rad). Primers used were: *K19* forward, 5′-CCAGGTCAGTGTGGAGGTGG-3′; reverse, 5′-TTGGCTTCGCATGTCACTCA-3′; *CSF2* forward, 5′-CACTGCTGCTGAGATGAATGA-3′; reverse, 5′-AATCTGGGTTGCACAGGAAG-3′; *SCYA4* forward, 5′-AAGCTCTGCGTGACTGTCCT-3′; reverse, 5′-GCTTGCTTCTTTTGGTTTGG-3′; *IL-8* forward, 5′-CTGCGCCAACACAGAAATTA-3′; reverse, 5′-ATTGCATCTGGCAACCCTAC-3′. Control reactions were performed by using primers for β-actin: forward, 5′- AGCCTCGCCTTTGCCGA-3′; reverse, 5′-CTGGTGCCTGGGGCG-3′ (Integrated DNA Technologies, Inc.). The mean ± standard error of measure (SEM) was calculated by using the Prism curve-fitting program (GraphPad Software).

Estrogen Receptor Binding Assay

U2OS-ERα stable cells were treated overnight with doxycycline (0.1 ng –1 μg/ml). Cells were then incubated for 2 hr at 37ºC with 20 nM [^{3}H] E2 (NEN, specific activity 87.6 Ci/mmol) in the absence or presence of 100-fold excess of unlabeled E_2. After washing with 0.1% bovine serum albumin in phosphate buffered saline, the cells were dissolved in SDS lysis buffer (0.5% SDS, 0.05 M Tris-HCl [pH 8.0], and 1 mM DTT). Specific binding was calculated as the difference between total and nonspecific binding and expressed as cpm/1.5 million cells.

Western Blotting Analysis

Total proteins (15 μg) from cells were separated with 4%–12% gradient Bis-Tris gels (Invitrogen). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Perkin Elmer) and incubated with anti-ERα (DAKO), anti-Phospho-ERα (Ser-118) (Cell Signaling), or anti-Phospho-ERα (Ser-167) (Cell Signaling) diluted 1:1000 in blocking buffer followed by anti-mouse IgG conjugated with horseradish peroxidase (PharMingen). An ECL detection system (Amersham Biosciences) was used to visualize the proteins.

Coimmunoprecipitation

Cells were treated with doxycycline for 18 hr and TNFα for 2 hr. Cells were lysed in RIPA buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl, and protease inhibitors), and extracts (500 μg) were incubated with a p50-NFκB, p65-NFκB (Upstate Biotechnology), or c-*jun* (Santa Cruz Biotechnology) antibody and protein G agarose overnight at 4ºC. Immunoprecipitated proteins were separated by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane. Blots were incubated with ERα antibody (DAKO) and detected with the ECL system (Amersham BioSciences).

RNA Interference

MCF-7 or MDA-MB-435 cells were plated in 4% charcoal dextran stripped FBS-DMEM/F-12 media and grown to 50% confluency. Cells were transfected with ERα ShortCut siRNA Mix or MBP shortcut siRNA mix as a negative control (New England Biolabs). After 48 hr, a second transfection was performed for a total of 4 days. Cells were treated with TNFα (5 ng/ml) for 2 hr, and then RNA or protein were prepared for qPCR and Western blots, respectively.

Plasmid Construction, Virus Production, and Cell Culture

Adenovirus expressing ERα was prepared as previously described (Paruthiyil et al., 2004). The lentiviruses were produced with PCR products containing the U6 promoter and a shRNA (Li et al., 2004) to two different regions of GRIP1. The primers used were: 5′-AAAACTGCAGAAAAAGGAATGTCCTGACCAACTTGGACCCTCTCTTGAAGGGTCCTAGTTGGTGAGGACATTCCGGTGTTTCGTCCTTTCCACAAG-3′ or 5′-AAAACTGCAGAAAAAGTGCAGAAGTCAGATGTATCCTCTATCTCTTGAATAGAGGTTACATCTCACTTCTGCACGGTGTTTCGTCCTTTCCACAAG-3′ and 5′-AAAAACTAGTAAGGTCGGGCAGGAAGAGGGC-3′. The two PCR products were subcloned into the pHRCMVPUROWSin18 vector and then transfected into HEK293 T cells along with pMD.G1 and pCMVDR8.91 (generously provided by Dr. Didier Trono, University of Geneva). After 48 hr and 72 hr, the culture medium containing the lentivirus expressing GRIP1 shRNA was harvested. U2OS-ERα cells were infected with a lentivirus expressing a scrambled shRNA (generously provided by Dr. Alex So, University of California, San Francisco) or sequentially with two different lentiviruses expressing GRIP1 shRNA. After infection, cells were selected with 3 μg/ml puromycin.

ChIP and Re-ChIP

Cells were treated as indicated in legends and then were cross-linked, washed, collected, and lysed as previously described

(Burakov et al., 2002; Kian Tee et al., 2004). Immunoprecipitations were performed overnight at 4ºC with anti-p50 NFκB, anti-p65 NFκB, anti-SRC-1 (clone 1135) (Upstate Biotechnology), anti-c-*jun* (H-79), anti-CBP (A-22), anti-N-CoR (C-20), anti-ERα (HC-20) (Santa Cruz Biotechnology), anti-flag M2 (Sigma-Aldrich), anti-GRIP1 (ab9261), anti-AIB1 (ab2782) (Abcam), anti-ERα (1D5) (DAKO), anti-ERα (MSERA11-UP50), anti-ERβ (6A12, 14C8 and 7B10) (GeneTex, Inc), and anti-Hsp90 (AC88) (StressGen). The −167 to +42 region of the *TNFα* gene was amplified with the primers 5′-CCCCGCGATG GAGAAGAAACCGAGA-3′ and 5′-GCTGGTCCTCTGCTGTCCTTGCT GA-3′. The primers used to amplify the *keratin 19* estrogen response element (ERE) (Choi et al., 2000) were: forward, 5′-TCCAGCCTGGGT GACAGAGC-3′; and reverse, 5′-TCCAAGTTCACCCCAACCTGA-3′. For re-ChIP experiments, after primary immunoprecipitation, cross-linked complexes were eluted from the immunoprecipitate and were reimmunoprecipitated with the second antibody as previously described (Metivier et al., 2003). The copy number of the samples was calculated by qPCR from a standard curve prepared by using a serial titration of the plasmid containing the TNF-RE. The final results were expressed as the percentages of the initial inputs.

DNA Pull-Down Assays

U2OS-ERα cells were induced with doxycycline for 18 hr and then treated with TNFα in the absence or presence of E_2. Nuclear extracts were prepared as described in the μMACS Streptavidin Kit protocol (Miltenyi Biotec) with the exception that the nuclei were not sonicated. A biotinylated synthetic oligonucleotide containing the TNF-RE (forward, 5′-Bio/GGTACCGAGCTCTTACGCGTGCACTACC GCTTCCTCCAGATGAGCTCATGGGTTTCTCCACCAAGCTCGAGAT CT-3′; reverse, 5′-AGATCTCGAGCTTGGTGGAGAAACCCATGAGC TCATCTGGAGGAAGCGGTAGTGCACGCGTAAGAGCTCGGTACC-3′) was annealed and incubated with μMACS Streptavidin MicroBeads. Proteins in the nuclear extracts were captured with MicroBeads, eluted, and separated on SDS-PAGE. Western blots were done with antibodies to ERα.

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