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PRINCIPAL INVESTIGATOR: Kendall W. Nettles
Geoffrey Greene, Ph.D.

CONTRACTING ORGANIZATION: The University of Chicago
Chicago, Illinois 60637

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6. AUTHOR(S)
Kendall W. Nettles
Geoffrey Greene, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Chicago
Chicago, Illinois 60637

E-Mail: knettles@uchicago.edu

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13. ABSTRACT (Maximum 200 Words)

Estrogen Receptor-alpha (ER) mediated inhibition of NF-kappaB contributes to the anti-inflammatory and protective effects of estrogen in bone, cardiovasculature, and breast cancer. Cross talk could be caused by direct or indirect association of these transcription factors, or by competition for other components of the transcriptional apparatus. In order to distinguish among these possibilities, we identified clonal variants of ER(+) MCF-7 breast cancer cells that either do (MCF-7 SI) or do not (MCF7 SS) display ER mediated inhibition of NF-kappaB transcriptional activity. Transient transfection of various coactivators intot he MCF-7SS cells revealed that only CBP and p300 were able to promote an inhibitory effect ofestradiolon NF-kappaB activity. Western Bolt analysis showed that CBP protein levels were reduced in this cell line relative to to the MCF-7SI cells. Both immunofluorescent microscopy and co-immunoprecipitation shoed an associated between ER and NF-kappaB in the MCF-7SI cells. CBP also immunoprecipitated with both ER and NF-kappaB.

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Annual Summary Training Report

Key Accomplishments

- Defined the ER ligand binding domain as required for physical association with NF-kappaB in immunoprecipitation.
- Identified ligand binding domain of ER as necessary for trans-repression of NF-kappB.
- Used chromatin immunoprecipitation to demonstrate that ER is recruited to the MCP-1 NF-kappaB response element in intact cells.
- Expressed and purified the ER LBD for crystallization.
- Completed subcloning of the CBP CH3 domain into a bacterial expression vector.
- Purified recombinant CH3 domain and the ER ligand binding domain.
- Obtained crystals of the complex of ER LBD with the CH3 domain in preparation for x-ray crystallography.

Training and Research Accomplishments Related to the Statement of Work (Statement of Work attached as Abstract).

Please note that any uncompleted tasks are due to the graduation of the Principal Investigator.

Task 1: To test the hypothesis that p160 coactivators multimerize, assemble onto each LBD of an ERα dimer, and are redundant for ERα, but not for NF-κB function.

1a. Construction and purification of GST-ER completed.

1b. RNAi against CBP was tested and proved effective in reducing CBP protein expression in cell lines.

1c. A series of deletion mutant ER constructs were used to identify the region of ER that interacts with NF-kappaB in an immunoprecipitation assay. These experiments clearly identified the ER ligand binding domain as necessary and sufficient for the association. A series of CBP deletion constructs were also used to identify the regions of CBP required for mediating suppressive cross-talk in a transient transfection assay. Deletion of the p160 interaction domain in the CBP c-terminus had no effect, as this construct was still able to facilitate an estradiol mediated suppression of NF-kappaB transcription in the MCF-7SS cells. Cos-7 cells were used to test for dominant negative effects of deletion CBP constructs on ER- NF-kappaB cross-talk. Deletion of the p65 interacting domain or the CBP CH3 domain allowed CBP to act in an dominant negative fashion, blocking the estradiol mediated suppression of NF-kappaB. The GST-ER-ligand binding domain was used to demonstrate a direct interaction between the ER and the CBP CH3 domain.

1d. The effects of CBP RNAi will be examined on NF-kappaB transcription were examined in preliminary experiments, which were inconclusive. Further work was not performed due to the graduation of the Principal Investigator.

1e. As detailed in last years report, this assay was replaced with a variety of other experiments designed to probe for associations.

Task 2: To test the hypothesis that coactivators binding directly to to ERα and NF-kappaB mediate the inhibitory interactions between them.

2a. Association of ER with HSP9O was not examined at the recommendation of my thesis committee.

2b. Luciferase assay developed and tested

2c. Transient transfection assays developed and completed. These experiments identified the regions of CBP required for ER mediated suppression of NF-kappaB, namely the p65 interaction region in the n-terminus, and the ER interaction region in the CH3 domain. Notably, the p160 interaction domain in the c-terminus was not required.

2d. A PCAF expression plasmid was obtained for another investigator.

2e. Overexpression of PCAF blocked the suppressive effects of estradiol in transient transfection assays, suggesting the competition between ER and PCAF for the CH3 domain may be the fundamental mechanism of repression. P65 DNA binding activity was measured with DNA gel shifts and MCF7 extracts, demonstrating that there was no loss of DNA binding activity associated with estradiol treatment.

Task 3: Examine the role of the CH3 domain of CBP in the ER-mediated inhibition of NF-kappaB, and the structural basis of the CH3-ER association.

1a. These experiments were not performed due to the graduation of the principal investigator.

1b. Binding of ER to the MCP-1 NFkappaB response element DNA sequence was demonstrated with the chromatin immunoprecipitation assay. Binding of PCAF will be examined during months 24-36.

1c-f. Crystals were obtained of the complex of ER with the CH3 domain, using the expression plasmid from taks 3-1c, and the purified CH3 protein from taks 3-1d . These were single crystals of the size 10x10x50 microns, which were not large enough to obtain data.

Introduction

In this work we find that ER and p65 both interact with CBP, and that the CH3 domain, but not the SRC-1 interaction domain of CBP is required for suppression of NFκB by ER. The CH3 domain binds ER and pCAF, suggesting that competition for this site might reduce the localization of pCAF to the MCP-1 enhancer. This reveals a novel role for the CH3 domain in ER function.

Report Body

ER suppresses MCP-1 through Inhibition of NFKB Transcriptional Activity.

MCP1 was identified as an estrogen repressed gene by RNA differential display (summarized in figure 1). The ER positive MCF-7 breast cancer line was treated with estradiol and cyclohexamide, an inhibitor of protein synthesis, in order to select genes that were directly regulated by ER (Figure 1 A). The suppression of MCP1 mRNA by estradiol was confirmed by northern blot analysis in these cells. Similar results were seen with the ER positive T47D breast cancer cell, but not in the ER negative AU-565 breast tumor cell line. The specificity of this effect in the MCF7 cells was also shown by competition with the ER antagonist ICI 182780, and by the ineffectiveness of androgen receptor and progesterone receptor agonists in suppressing MCP-1 mRNA levels (data not shown).

MCP-1 is a gene induced by NFκB and that lacks a defined estrogen response element. In order to investigate the role of NFκB in the MCF7 cells, a physiological inducer of NFκB, TNFα, was used rather than cyclohexamide. Estradiol was also effective in suppressing TNFα-induced MPC-1 mRNA (Figure 1A). The role of the "A2" NFκB binding element from the distal enhancer region of the MCP-1 gene was examined in a luciferase reporter assay. This reproduced the suppressive effects of E2 on transcription of the MCP-1 gene in several cell lines (figure 1E). Deletion of the A2 sequence eliminated TNFα-induced MCP1-luciferase activity (Vandana Sharma, data not shown), further supporting the importance of NFκB in the suppressive effects of estradiol on the MCP1 gene. Co-transfection of ER allowed an estradiol dependent suppression of MCP1-luciferase activity in Cos-7 and Ishikawa cell lines, which do not express ER natively (figure 1E). This further supports the role of ER in the suppressive effects of estradiol. A radiolabeled oligonucleotide comprised of the MCP-1 A2 response element showed TNFα-dependent binding to p65 from MCF-7 extracts (Figure 2). Combined treatment with TNFα and estradiol had no effect on the DNA binding levels of p65, suggesting that transcriptional suppression occurs through another mechanism.

The MCP1 gene could be regulated at a number of different levels by estradiol. This was investigated by measuring the rate of MCP-1 mRNA turnover and the rate of transcription. Actinomycin treatment of MCF7 cells was used to block transcription, allowing a measure of mRNA degradation over time. MCP-1 mRNA, relative to GADPH, showed the same rate of degradation following TNFα treatment regardless of the presence of estradiol (figure 1B). This suggests that the gene is regulated at the transcriptional level by estradiol. This was confirmed with the nuclear run-on assay, a measure of the level of transcription. Estradiol treatment significantly reduced the number of transcripts induced by TNFα. As also shown in figures 1A-B, there was no measurable MCP-1 transcript from the vehicle treated extracts.

The activation and nuclear translocation of NFκB were also unaffected by estrogen treatment. The upstream activator of NFκB, IκBα, was not transcriptionally regulated by estradiol treatment, as shown by a measure of its mRNA over time. The cyclohexamide experiments also suggest that no other protein synthesis was required. The nuclear translocation of p65 was examined by immunofluorescent staining, and was equivalent with or without estradiol treatment in the TNFα-induced MCF7 cells (figure 3. These data suggest that MCP-1 transcription is regulated through NFκB, and that ER inhibits it at that level.

CBP/p300 Are Sufficient for ER to Suppress NFKB

ER could interact with NFκB by competing for lin 128 tors, or through physical associations, which could be either direct, or indirect in a complex with other cofactors. Over-expression of coactivators has been

shown to eliminate cross-talk between other transcription factors. However, such data has been interpreted as supporting both the competition and the association models (Harnish et al., 2000; McKay and Cidlowski, 2000; Sheppard et al., 1998; Speir et al., 2000; Webster and Perkins, 1999). A more rigorous test of the physical association model would be to induce the effect in a system that lacks it. That is, adding more of a limiting factor for which there is competition would eliminate cross-talk, but would never stimulate it to occur. The formation of an inhibitory complex is only consistent with the physical association model. To test this, we made use of clonal variants of the MCF-7 cell line. The MCF-7ES line (Estrogen Sensitive) was selected for growth sensitivity to estradiol, and does not show a suppressive effect of estrogen on MCP-1 transcriptional activity. The MCF7EI (estrogen insensitive) cell line was selected for growth in charcoal-dextran stripped media, and shows the suppressive effects in transient transfection (figure 1E), and by real time PCR analysis of the native MCP-1 mRNA (data not shown).

We examined the role of coactivators known to interact with NFκB and ER, including the SRC1-3, Trap220, pCAF, and CBP/p300. Transfection of increasing amounts of CBP expression plasmid into the MCF7-ES cells produced an estradiol

dependent suppression of NFκB transcriptional activity (figure 3A). Among the other coactivators tested, only the CBP homologue p300 showed the same effect (data not shown). This suggests that CBP protein levels contribute to the tissue specificity of this effect. Whole cell extracts of the MCF-7 variants showed that the MCF7-ES cells, which do not show suppression, have reduced levels of CBP protein compared to the MCF-EI cells (figure 3B).

ER and p65 physically associate

Immunofluorescent staining suggested that ER and p65 colocalize in the MCF7-EI cells. TNFα treatment alone induced nuclear translocation of p65, shown in red, and minimal co-localization with ER, shown in the merged channel as yellow (figure 3D). In contrast, combined treatment with estradiol induced a large increase in co-localization, suggesting that the colocalization is ligand dependent in the appropriate cellular context. The physical association of ER and p65 was examined by immunoprecipitation of native p65 protein from whole cell extracts of MCF-7SI cells. A western blot for ER showed that TNFα treatment, but not estradiol was required for interaction, and that TNFα plus estradiol treatment showed similar levels of interaction as TNFα alone (figure 3C). The estradiol independent nature of this association may reflect the loss of ER-heat shock protein interactions, which are highly unstable in in vitro assays (data not show; David Toft, personal communication

The ER domain requirements for suppression of NFκB were examined by transfection of a series of mutants into Cos-7 cells. The n-terminal A/B domain, the DNA-binding domain, and the hinge domain were all dispensable for the suppressive effects of ER (figure 4). In contrast, loss of the LBD, or a mutation of helix 12 that destroys the AF2 binding pocket completely abrogated the suppressive effects of ER. The domain requirements for ER to association with p65 were tested by co-transfection into Cos-7 cells, followed by immunoprecipitation. The ER-LBD was found to be required for interaction with p65, as there was no detectible interaction in its absence. In contrast, deletion of the DNA binding domain or of the hinge domain had no effect on the physical association. The western blot data also shows that the loss of suppression in the transient transfection experiment is not due to differences in protein expression. While the LBD is required for transcriptional repression and association with p65, it is not sufficient for the association. Thus GST fused to the ER-LBD was not able to pull-down in vitro translated p65 (figure 5B). This is consistent with the formation of a ternary complex with CBP as the mechanism of association.

Domain requirements for CBP and role of P/CAF:

Both ER and NFκB can associate with CBP through multiple domains, including the n-terminus and via the p160 proteins, which interact with the c-terminus. We first examined the requirement for the p160 interaction domain by transfecting CBP 1-1899 into the MCF-7ES cells. This construct, missing the c-terminal p160 interaction domain, showed the same effectiveness as full-length CBP in allowing ER to suppress MCP-1 transcriptional activity (figure 5A). If there is a ternary complex between ER, p65, and CBP, then a portion of CBP that binds only ER should act as a dominant negative construct, relieving the suppressive effects of estradiol. This was tested by transient transfection of CBP deletion constructs into Cos-7 cells There has

recently been identified an interaction between ER and the CBP CH3 domain, located in the last 200 amino acids of the CBP 1-1899 construct (Fan et al., 2002). Transfection of the CH3 domain, in CBP 1457-1899 acted as a dominant negative construct (figure 5C). The GST fusion with ER-LBD was able to interact with CBP 1457-1899, suggesting that this direct interaction may account for the suppressive effects of estradiol on NFκB (figure 5B). In addition to the interaction of p65 with the CBP n-terminus, an association of p65 with the CH1 domain is required for full activity (Zhong et al., 1998). Deletion of this domain, CBPΔ142-705, allowed CBP to act as a dominant negative construct, eliminating the suppressive effects of estradiol without compromising the induction by TNFα (figure 5C), as predicted of a construct which should bind ER but not p65. CBP 1-1899 contains interaction sites for both p65 and ER, and did not act as a dominant negative, but rather allowed the suppressive effects of estradiol on NF-κB activity (figures 5A and 5C)

The mechanism of suppression is suggested by an examination of other proteins that interact with the CBP CH3 domain, notably pCAF. The HAT domain and the CBP interaction domain of pCAF are required for NFκB transcriptional activity(Brockmann et al., 2001; Sheppard et al., 1999). In contrast, CBP coactivates NFκB equally well in the absence of functional HAT domain. The E1A 12S protein inhibits NFκB activity (Gerritsen et al., 1997) through interaction with the CBP CH3 domain (Sheppard et al., 1999), further supporting the importance of this region in NFκB transactivation. Thus ER could suppress NFκB through competing with pCAF for this binding site on CBP. To test this, pCAF was transfected into Cos-7 cells, showing a dose-dependent reversal of the inhibitory effects of ER (figure 5D).

Reportable Outcomes

This work was presented as an abstract at the Era of Hope Annual Meeting. The manuscript is in the final stages of preparation for publication. The Principal Investigator obtained two awards based on this work:

Best Thesis 2003, Committee on Cancer Biology, The University of Chicago
Elaine Erhman Award for Research in Cancer Biology, 2002. Awarded yearly to a senior graduate student at the University of Chicago

Conclusions

Suppression of MCP-1 gene expression represents an important therapeutic target for protection from cardiovascular disease and malignant progression in breast cancer. In the MCF-7 cell, we established that ER suppression of MCP-1 mRNA occurs at the level of transcriptional regulation, via suppression of the transcriptional activity of NF-κB. ER did not influence the expression of the upstream regulator or NF-κB, and did not effect the DNA binding capabilities of TNF stimulated p65 in MCF-7 extracts, nor the nuclear localization of native p65 protein.

In the MCF-7 cell line, a physical association between native ER and p65 proteins may underlie their suppressive interactions. Immunoflourescent co-localization, immunoprecipitation, and in pilot experiments, the recruitment of ER to the MCP-1 NF-κB response element DNA support this model. A mammalian two hybrid experiment (Wyeth) suggested that CBP coactivator may bridge the association between ER and NF-κB. This work demonstrates that native CBP protein co-immunoprecipitates with both ER and p65. Evidence for a ternary complex derives from the effects of CBP transfection into a clonal variant of MCF-7 that does not allow estradiol mediated suppression of MCP-1 trancriptional activity. If ER and NF-κB competed for limiting amounts of CBP, then addition of more CBP could relieve suppression, but could never induce a suppressive effect. Increasing amounts of CBP allowed ER to suppress MCP-1 luciferase activity.

Surprisingly, the p160 interaction domain of CBP was dispensable for suppression of MCP-1 activity by ER, as CBP1-1899 was equally efficatous at allowing the suppression as the wild type CBP. We reproduced the recent report that ER the ER-LBD interacts directly with the CBP CH3 domain, located in the last 150 amino acids of the CBP 1-1899 construct, suggesting that this domain might be important for the suppressive effects. Indeed, the CBP CH3 domain in isolation acted in a dominant negative fashion to relieve the suppressive effects of ER on NF-κB activity. Deletion of the p65 interaction domain in CBP also produced a dominant negative construct, further supporting the model that recruitment of ER to a CBP/p65 complex inhibits NF-κB activity.

The domain requirements for suppression were carried out using native p65 protein in the Cos-7 cell, which

does not possess native ER. Deletion of the n-terminus, DNA binding domain, or hinge had no effects on the ER/p65 physical association, or transcriptional suppression of NF-κB, while deletion of the ER LBD abrogated by the physical and functional interactions. This result is consistent with the interaction of ER with the CH3 domain of CBP as accounting for suppressive effects.

It is not intuitively obvious how a well characterized transcriptional coactivator might facilitate suppression of transcription. A similar function was recently ascribed to the Grip p160 coactivator in mediating Glucocorticoidal suppressive effects on NF-κB, through a previously uncharacterized domain of Grip. In this case, the CH3 domain has been previously identified as the interaction site on CBP for the P/CAF coactivator, a HAT protein with an essential role on NF-κB mediated transcriptional activation. This suggests a simple model in which ER displacement of P/CAF from the p65/CBP complex reduces the essential HAT activity, and decreases transcriptional activation.

This hypothesis was supported by the reversal of ER mediated suppression following transfection of P/CAF, suggesting that the cellular ratio of CBP to P/CAF may be an important determinant of transcriptional integration.

Please note that any uncompleted tasks are due to the graduation of the Principal Investigator.

References

Brockmann, D., Lehmkuhler, O., Schmucker, U., and Esche, H. (2001). The histone acetyltransferase activity of PCAF cooperates with the brahma/SWI2-related protein BRG-1 in the activation of the enhancer A of the MHC class I promoter. Gene *277*, 111-120.

Fan, S., Ma, Y. X., Wang, C., Yuan, R. Q., Meng, Q., Wang, J. A., Erdos, M., Goldberg, I. D., Webb, P., Kushner, P. J., *et al.* (2002). p300 Modulates the BRCA1 inhibition of estrogen receptor activity. Cancer Res *62*, 141-151.

Gerritsen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., and Collins, T. (1997). CREB-binding protein/p300 are transcriptional coactivators of p65. Proc Natl Acad Sci U S A *94*, 2927-2932.

Harnish, D. C., Scicchitano, M. S., Adelman, S. J., Lyttle, C. R., and Karathanasis, S. K. (2000). The role of CBP in estrogen receptor cross-talk with nuclear factor-kappaB in HepG2 cells. Endocrinology *141*, 3403-3411.

McKay, L. I., and Cidlowski, J. A. (2000). CBP (CREB binding protein) integrates NF-kappaB (nuclear factor-kappaB) and glucocorticoid receptor physical interactions and antagonism. Mol Endocrinol *14*, 1222-1234.

Sheppard, K. A., Phelps, K. M., Williams, A. J., Thanos, D., Glass, C. K., Rosenfeld, M. G., Gerritsen, M. E., and Collins, T. (1998). Nuclear integration of glucocorticoid receptor and nuclear factor-kappaB signaling by CREB-binding protein and steroid receptor coactivator-1. J Biol Chem *273*, 29291-29294.

Sheppard, K. A., Rose, D. W., Haque, Z. K., Kurokawa, R., McInerney, E., Westin, S., Thanos, D., Rosenfeld, M. G., Glass, C. K., and Collins, T. (1999). Transcriptional activation by NF-kappaB requires multiple coactivators. Mol Cell Biol *19*, 6367-6378.

Speir, E., Yu, Z. X., Takeda, K., Ferrans, V. J., and Cannon, R. O., 3rd (2000). Competition for p300 regulates transcription by estrogen receptors and nuclear factor-kappaB in human coronary smooth muscle cells. Circ Res *87*, 1006-1011.

Webster, G. A., and Perkins, N. D. (1999). Transcriptional cross talk between NF-kappaB and p53. Mol Cell Biol *19*, 3485-3495.

Zhong, H., Voll, R. E., and Ghosh, S. (1998). Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. Mol Cell *1*, 661-671.

Statement of Work

Task 1: to test the hypothesis the p160 coactivators multimerize, assemble onto each LBD of an ERα dimer, and are redundant for ERα,but not NF-κB function.

a. Develop and purify ER and GST-ER-LBD and dimerization defective GST-ER-LBD proteins, including mutagenesis of the GST-eR-LBD plasmid (months 1-6).
b. Develop and characterize p160 antisense nucleotides in cell culture, including northern and western blot analysis fot he p160 family members. (months 1-12).
c. Perform pull-down assays. (months1-18).
d. Perform transient transfection assays with antisense nucleotides (months 12-24).
e. Confirm protein interactions with yeast two hybrid system (months 24-36).

Task 2: To test the hypothesis that coactivators binding directly to to ERα and NF-kappaB mediate the inhibitory interactions between them.

a. Purify ER from MCF-7 extracts under conditions that allow retention of chaperone complex,followedby western blot analysis for ER, p65, and HSP90 (months 1-12).
b. Develop luciferase reporter with estrogen and NF-kappaB response elements. (months 1-6)
c. Perform transient transfection assays with transcriptional coactivator expression plasmids (months 6-24), verifying ER and p65 protein expression by western blot.
d. Perform transient transfection of ASC-1 and PCAF, determination of NF-kappaB and ER DNA binding activity in cell extracts (months 24-36).

Task 3: Examine the role of the CH3 domain of CBP in the ER-mediated inhibition of NFlkappaB, and the structural basis of the CH3 ER association.

a. Test if in vitro translated PCAF competes away CH3 CBP binding to GST-ER (months 12-24).
b. Examine if ER competed for PCAF binding to the NF-KappaB enhancer of the MCP-1 gene using chromatin immunoprecipitation (months 12-36).
c. Clone the CH3 domain of CBP into a bacterial expression vector (months 12-14).
d. Express and purify CH3 CBP and ER (months 12-16).
e. Cocrystallize ER and CH3 CBP (months 16-24).

Crystal structure determination (months 24-36).

Please note that any uncompleted tasks are due to the graduation of the Principal Investigator.

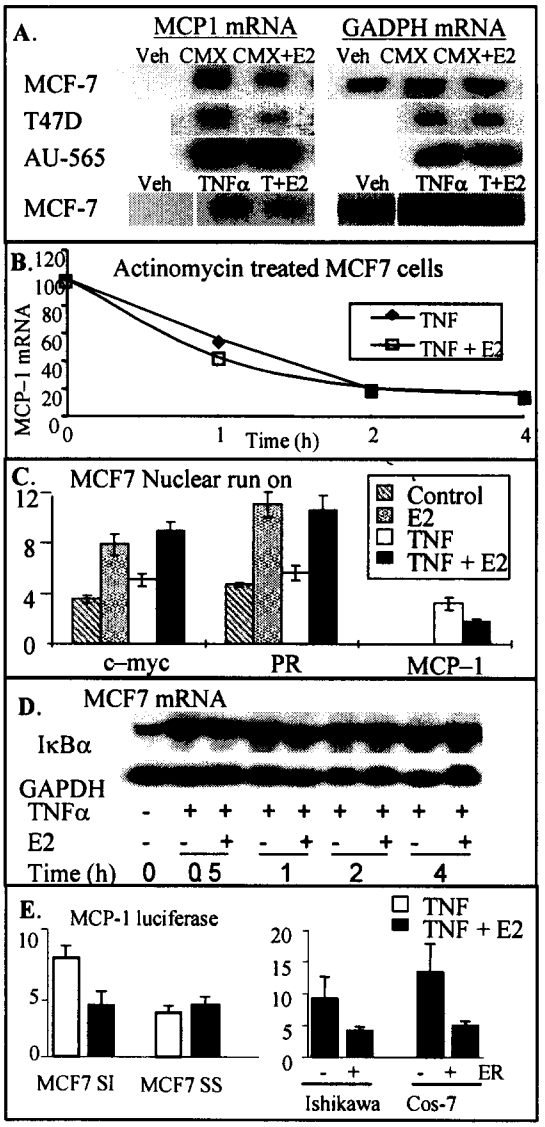

Figure1. Estradiol suppresses MCP1 Transcriptional Activity. **A**, Northern blot of mRNA extracted from breast cancer cell lines. They were treated with either cyclohexamide or TNF for 0.5 hour, followed by E2 or vehicle for 4 hours. The ER negative AU-565 cell line showed no suppressive effects of E2. **B**, MCP1 mRNA, normalized to GADPH mRNA, demonstrates the same rate of degradation with or without E2 treatment. Actinomycin was used to arrest transcription. **C**, nuclei were isolated and subject to *in vitro* run-on transcription to measure levels of partially transcribed RNA. MCP1, but not c-myc or PR mRNA is induced by TNF and suppressed by E2. **D**, The upstream activator of NFκB is stimulated by TNF treatment, and unaffected by E2 treatment. **E**, Fold induction of MCP1 luciferase activity. We identified clonal variants of MCF7 cells that either do, or do not show suppressive effects of E2. Right panel, Transfection of ER allows suppression of MCP1 transcriptional activity in other cell lines.

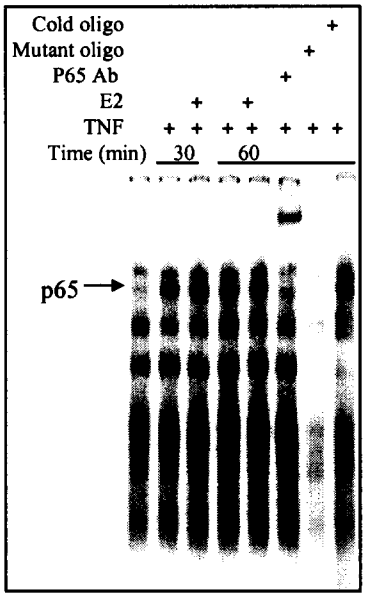

Figure 2. Gel Shift of radiolabelled NFκB response element oligonucleotide from MCP1 gene. MCF-EI cells were treated for 30 or 60 minutes and then made into nuclear extracts. Following 15 pre-incubation with the probe, the extracts were electrophoresed for 1.5 hours at 10C. The p65 band was induced by TNF and shifted by a p65 antibody. There was no effect of E2 treatment on the DNA binding of p65. Incubation with excess cold oligo prevented the gel shift, while excess mutant cold oligo had no effect.

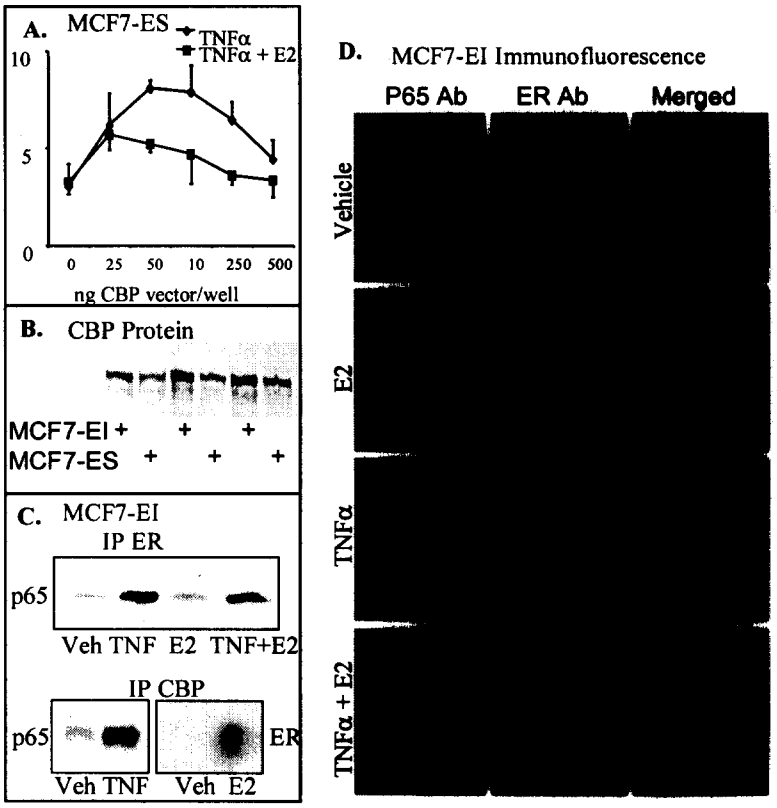

Figure 3. Interaction of CBP, ER and NFKB **A,** Fold induction MCP1 luciferase activity in MCF7-ES cells, showing no suppression with E2 in the absence of added coactivator. Transient transfection of increasing amounts of CBP expression plasmid allows E2 mediated suppression of MCP1 transcriptional activity. **B,** Western Bolot of CBP protein levels. Levels are reduced in the MCF-ES cells, that do not show suppression, relative to MCF-EI cells that do show E2-mediated suppression of NFκB. Whole cell extracts were normalized for protein content. Equal loading was verified with ponceau staining. Shown is a experiment in triplicate. **C,** Immunoprecipitation of MCF-EI extracts, treated with ligands for 30 minutes before cell lysis. Immunoprecipitation of ER and western blot for p65 demonstrates TNF-dependence. Immunoprecipitation of CBP and western blot for p65 or ER shows appropriate ligand dependence. **D,** MCF-EI cells were grown on cover-slips, treated for 30 minutes with ligands, methanol fixed, and stained for immunofluorescent microscopy. Left column, p65 translocates to nucleus upon TNF treatment. Right column, merge of ER and p65 signals demonstrates requirement for both ligands for efficient colocalization.

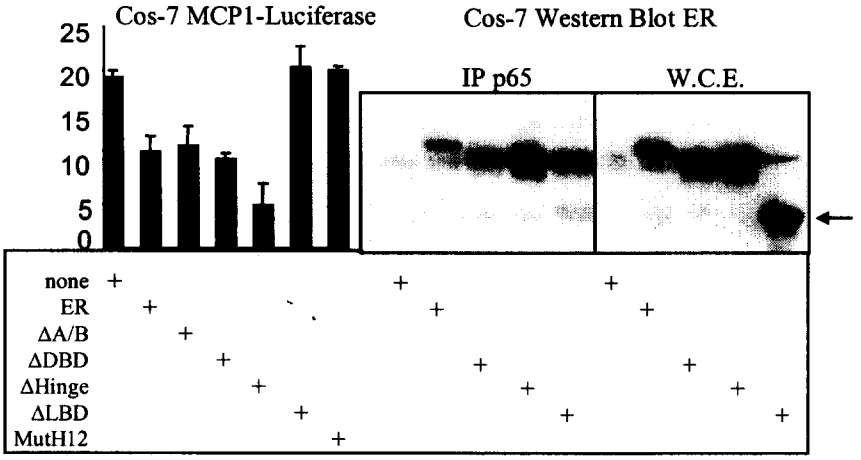

Figure 4. ER LBD required for repression and association with p65. Left panel, Cos cells were transfected with MCP1 luciferase reporter and ER expression plasmids. The next day, cells were treated with TNF+E2 for 6hours. The 20-fold induction was reduced 50% by transfection of ER. Deletion of the n-terminal A/B transactivation domain, the DBD or the hinge between DBD and LBD had no effect on suppression. Deletion of the LBD or point mutation of helix 12 blocked suppression. **Right panel**, Cos cells were transfected with p65 and ER expression plasmids as indicated. After 48 hours, cells were treated for 30 minutes with TNF+e2 and lysed for protein extraction. Only the ΔLBD was unable to interact with p65, though it was expressed at high levels in the whole cell extract (arrow).

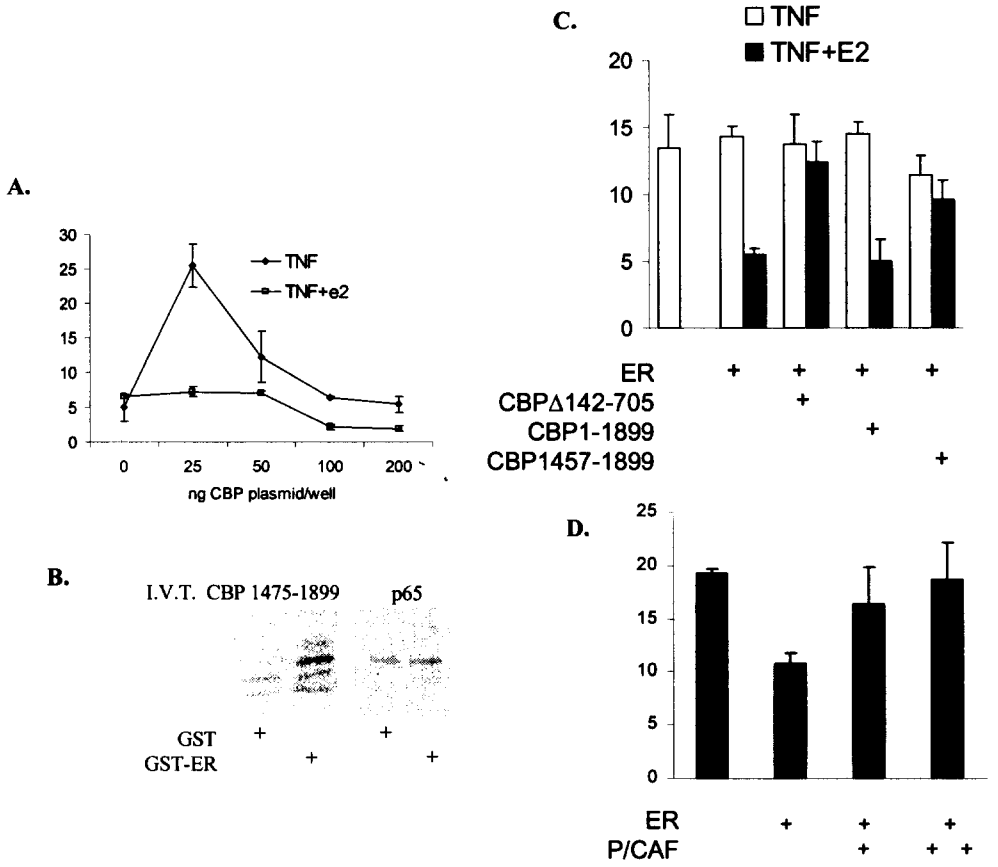

Figure 5. CBP domain analysis and role of P/CAF a, transfection of MCP1 luciferase reporter into MCF-ES cells demonstrates that CBP 1-1899, missing the p1260 interaction domain, allows E2 to efficiently suppress NFκB trancriptional activity. **B,** GST-ER LBD interacts with the in vitro translated CH3 domain of CBP, but not with p65. Deletion of the p65 interaction domain of CBP, located in the n-terminus, creates a dominant negative construct that reversed the inhibitory effects of estradiol on MCP1 transcriptional activity. The CH3 domain is sufficient for dominant negative activity. **D,** PCAF reverses suppressive effects of E2 on MCP-1 transcriptional activity. PCAF has been shown by others to bind the CH3 domain of CBP, and be required for p65 activity.