Estrogen Receptor-α Binds p53 Tumor Suppressor Protein Directly and Represses Its Function

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Cancer Institute, Buffalo, New York 14263 and the Various observations have alluded to the potential for a cross-talk between p53-response elements. On the other hand, estrogen receptor-

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Estrogen receptor-α (ERα) promotes proliferation of breast cancer cells, whereas tumor suppressor protein p53 impedes proliferation of cells with genomic damage. Whether there is a direct link between these two antagonistic pathways has remained unclear. Here we report that ERα binds directly to p53 and represses its function. The activation function-2 (AF-2) domain of ERα binds directly to p53 and represses its function. The activation function-2 necessary for the interaction. Knocking down p53 and ERα by small interfering RNA elicits opposite effects on p53-target gene expression and cell cycle progression. Remarkably, ionizing radiation that causes genomic damage disrupts the interaction between ERα and p53. Ionizing radiation together with ERα knock down results in an additive effect on transcription of endogenous p53-target gene p21 (CDKN1A) in human breast cancer cells. Our findings reveal a novel mechanism for regulating p53 and suggest that suppressing p53 function is an important component in the pro-proliferative role of ERα.

As a tumor suppressor, p53 plays a central role in cellular processes such as cell cycle arrest, apoptosis, senescence, and differentiation (1, 2). Although these functions of p53 are essential to prevent cells from becoming cancerous, left uncontrolled, they can lead to consequences deleterious to normal cells. Mutations in the p53 gene or aberrations in the mechanisms to balance p53 function pave the way to tumorigenesis (3). p53 elicits its biological functions left uncontrolled, they can lead to consequences deleterious to normal cells. p53 elicits its biological functions mainly by functioning as a transcriptional regulator of various cellular genes (4–8). ERα is a transcriptional regulator that is recruited to the promoter regions of target genes directly through binding to estrogen response elements (EREs) or indirectly through other DNA-binding factors, such as AP1 and Sp1 (7, 9). The opposing functions of p53 and ERα, while stringently controlled in normal cells, are likely disrupted in cancer cells. Various observations have alluded to the potential for a cross-talk between p53 and ERα signaling pathways. For example, in murine models, early exposure to 17β-estradiol (E2) and progesterone to mimic pregnancy induced nuclear p53 enabling resistance to carcinogenesis by blocking proliferation of apparently ERα-positive cells (10). In breast cancer cells, increased expression of ERα led to elevated levels of p53 and MDM2, an inhibitor of p53 function (11), whereas overexpression of MDM2 enhanced the function of ERα (12). However, whether there is a direct link between the p53 and ERα pathways has remained unclear. To address this important issue, we investigated whether ERα directly interacts with p53 and affects its function.

EXPERIMENTAL PROCEDURES

Cell Culture and Irradiation—MCF7 cells and Saos2 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen) or 10% dextran-charcoal-treated FBS at 37 °C under 5% CO2. MCF7 cells were irradiated at a dose of 12 grays in a γ-cell 40, S/N 20 irradiator. Control cells were mock-irradiated.

Plasmids—The −1265 PCNA-luc reporter plasmid was described previously (13). pRc/CMV hp53, plasmids expressing wild type and mutant glutathione S-transferase (GST)-p53 proteins, and −2326 p21-luc were kindly provided by A. J. Levine, T. Shenk, and W. El-Deiry, respectively. The pCR3.1-based hERα expression plasmids (ER, ER 179C, ER C201H/C206H, ER L539A) and pRST7N282G were from C. Smith. pCR3.1-ERΔ283−595 was constructed by transferring the BamHI-HindIII fragment from pRST7N282G to pCR3.1 linearized with BamHI and HindIII.

Transient Transfection and Inhibition of Endogenous ERα and p53 Expression by siRNA—Saos2 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% dextran-charcoal-treated FBS were transfected with −1265 PCNA-luc, p53, and wild type and mutant ERα expression plasmid using Lipofectamine 2000 (Invitrogen). To ascertain the specificity of siRNAs used, the mRNA and the protein levels as well as effects on functional targets of the targeted protein were monitored.

Quantitative Real-time (qRT) PCR—Total RNA from MCF7 cells was isolated using the “Absolutely RNA Miniprep Kit” (Stratagene). For analyzing transcription of p21, 1 µg of total RNA was reverse-transcribed in 20 µl of reaction using the “SuperScript III First-Strand Synthesis System” (Invitrogen). One µl of the resulting cDNA was used in a total volume of 25 µl of PCR reaction. Real-time PCR was carried out in an Applied Biosystems Prism 7900 Sequence Detection System using TaqMan PCR master mixture, probes, and primers (Applied Biosystems). The relative mRNA levels in MCF7 cells transfected with specific versus NS siRNA were calculated using the ΔΔCt method with the endogenous β-actin mRNA as control.

Immunoprecipitation—MCF7 cells irradiated or mock-irradiated were washed twice with phosphate-buffered saline, lysed in NENT buffer (100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 20 mM Tris-HCl, pH 8.0) containing 1 mM halde peptide inhibitor mixture (Pierce). The extracts were cleared by centrifugation for 10 min at 14,000 rpm at 4 °C and 2.5 µg of each lysate was preclarified with agarose-conjugated mouse IgG (2 h, 4 °C) and subsequently incubated with agarose-conjugated mouse IgG or mouse monoclonal p53 antibody (DO-1, Santa Cruz Biotechnology) overnight at 4 °C. Agarose beads with immunoprecipitated proteins were washed four times with NENT buffer, boiled in SDS sample buffer, and resolved by SDS-PAGE followed by Western blotting using ECL method (Amersham Biosciences).

Antibodies for Immunoblotting— Immunoblotting was performed as described previously (14) with minor modification with the following antibodies: p53 (DO-1), and ERα (HC-20 and D-12), from Santa Cruz Biotechnology), and β-actin (A2066) from Sigma.

Chromatin Immunoprecipitation (ChIP)—ChIP assays were performed on MCF7 and Saos2 cells using the ChIP kit (Upstate Biotechnology) as per the manufacturer’s instructions with minor modifications. In the case of Saos2 cells, 24 h prior to cross-linking, cells were transfected with p53 and ERα expression plasmids either individually or in combination. DNA-p53-ERα complexes were immunoprecipitated using antibodies against p53 (DO-1) or ERα (HC-20) (Santa Cruz Biotechnology). DNA after decross-linking, and purification was subjected to PCR with AccuPrime TaqDNA polymerase
RESULTS AND DISCUSSION

ERα Binds to p53—We used communoprecipitation assay to analyze association between endogenous ERα and p53 proteins in MCF7 (human breast cancer cells containing wild type ERα and wild type p53) cells. ERα was detected by immunoblotting in the cell lysate immunoprecipitated by p53 antibody (Fig. 1A). As genomic damage is known to activate p53 (15) and affect its interaction with proteins such as MDM2 and p53, we investigated whether the ERα-p53 interaction is sensitive to genomic damage. When MCF7 cells were exposed to γ-radiation, the interaction between endogenous ERα and p53 was disrupted (Fig. 1A). A GST-p53 pull-down assay showed that p53 bound to in vitro-translated wild type ERα, A/B domain deletion mutant ERα Δ1–179, and DNA-binding domain mutant ERα C201H/C205H (Fig. 1B, lanes 3–5), whereas ERα Δ283–595 was unable to bind to p53 (Fig. 1B, lane 6), indicating that the 283–395 region containing the ligand-binding and activation function-2 domains is necessary for binding to p53. A similar experiment with different GST mutant-p53 proteins mapped the interaction domain of p53 to the 75 amino acids (319–393) at the COOH terminus (Fig. 1A). This intriguining that the COOH terminus that is extensively modified in a stress-dependent p53 to the 75 amino acids (319–393) at the COOH terminus (Fig. 1A) with different GST-mutant p53 proteins mapped the interaction domain of ERα-p53 interaction is sensitive to genomic damage. When MCF7 cells were exposed to γ-radiation, the interaction between endogenous ERα and p53 was disrupted (Fig. 1A). A GST-p53 pull-down assay showed that p53 bound to in vitro-translated wild type ERα, A/B domain deletion mutant ERα Δ1–179, and DNA-binding domain mutant ERα C201H/C205H (Fig. 1B, lanes 3–5), whereas ERα Δ283–595 was unable to bind to p53 (Fig. 1B, lane 6), indicating that the 283–395 region containing the ligand-binding and activation function-2 domains is necessary for binding to p53. A similar experiment with different GST mutant-p53 proteins mapped the interaction domain of p53 to the 75 amino acids (319–393) at the COOH terminus (Fig. 1A). This intriguining that the COOH terminus that is extensively modified in a stress-dependent p53 to the 75 amino acids (319–393) at the COOH terminus (Fig. 1A) with different GST-mutant p53 proteins mapped the interaction domain of ERα-p53 interaction.

Interaction of ERα with p53 Bound to Target Gene Promoters in Vivo—As a first step toward investigating how the ERα-p53 interaction affects the transcription of p53-target genes, we used the ChIP assay to analyze their interaction in vivo on a p53-target gene, p21 (CDKN1A), in MCF7 cells. Interaction at two p53 sites (5′ site, –2.2 kb and 3′ site, –1.3 kb) (19) was assayed along with a control nonspecific region (–4.3 kb) in the promoter. As expected, p53 bound to both the 5′ and 3′ sites in the non-irradiated (Fig. 2A, lane 3) and in the γ-irradiated cells (Fig. 2A, lane 4). In the non-irradiated cells, ERα was also bound to both the 5′ and 3′ sites (Fig. 2A, lane 7). However, when cells were subjected to genomic damage by ionizing radiation, consistent with the immunoprecipitation data (Fig. 1A), ERα binding to both sites was reduced considerably (Fig. 2A, lane 8 and quantitative ChIP data in supplemental Fig. S1B). A similar pattern (data not shown) of p53 and ERα binding was observed on the p53-response element of the PCNA gene, another transcriptional target of p53 (20). Immunoblotting analysis showed that the ERα expression was unaffected, and the p53 level, as expected, was increased by irradiation (Fig. 2B). Therefore, the reduced binding of ERα to p53 on the p21 and PCNA promoters in response to ionizing radiation is not because of altered protein levels. In MCF7 cells where p53 was knocked down, ERα was unable to interact with the p21 promoter (Fig. 2C). Similarly, in p53–/– Saos2 cells, ERα was able to bind to the p53-target promoter only when exogenous p53 was expressed (Fig. 2D; compare lanes 2 and 4). These results demonstrate that ERα is accessing the promoter via the p53 bound to DNA. Furthermore, E2 augments the ERα-p53 interaction, although it is not necessary for interaction (Fig. 2F). Consistent with such an effect of estrogen on the interaction, transcription of p53-target gene p21 is also reduced in the presence of E2 (Fig. 2F). This data along with the finding from a global gene expression profiling in E2-treated MCF7 cells (31) suggest that E2 has a direct repressive effect on p21 transcription. Interestingly, early exposure of rodents to pregnancy levels of E2 and progesterone leads to nuclear accumulation of p53 leading to p53-dependent response to exposure to chemical carcinogens and radiation (21). Besides, E2 has been reported to increase p53 gene expression in MCF7 cells (22), and p53, on the other hand, transcriptionally up-regulates ERα (23). These observations along with our data showing mutual regulation of ERα and p53 levels (Fig. 3D) suggest that delicate mechanisms are at play in balancing the antagonistic functions of these proteins.

ERα Represses Transcriptional Activation by p53—Having observed an ERα-p53 interaction in vivo on the p53 response element of endogenous p53 target genes promoters, we analyzed the functional consequences of this interaction. In a transient transfection assay in Saos2 cells, p53, as expected (13), activated transcription of the PCNA-luciferase reporter with wild type p53-binding site (Fig. 3A) but not the reporter with a mutant p53-binding site (data not shown). Wild type ERα repressed transcriptional activation of the PCNA promoter by p53. However, two transcriptionally defective ERα mutants (ER L539A, a coactivator–binding mutant, and ER Δ283–595, a deletion mutant
that lacks the entire C terminus containing the activation function-2 domain) were not effective repressors of transcriptional activation by p53. Similar levels of p53 and ERα/H9251 proteins in cells transfected with p53 and ERα/H9251 expression constructs either alone or in combination (supplemental Fig. S4) indicate that repression was due to interaction of ERα with p53 bound to the PCNA promoter and not as a consequence of altered p53 or ERα expression.

Unlike in the case of antagonism between GR and p53 where p53 was sequestered in the cytoplasm (24), the repression of p53 function by ERα/H9251 is not due to unavailability of p53 in the nucleus but rather is a consequence of, as shown by the ChIP assay (Fig. 2), interaction of ERα/H9251 with p53 bound to the endogenous target gene promoter.

As ectopic expression of ERα can repress the transcriptional transactivation by p53 in transfected Saos2 cells, we examined the impact of knocking down endogenous ERα on p53 activity in MCF7 cells. Analysis of mRNA by qRT-PCR showed that transcription of the endogenous p21 gene started to increase as early as 8 h after transfecting ERα siRNA (Fig. 3B) suggesting that increase in...
p21 transcription is not a consequence of cell cycle arrest. Furthermore, transcrip-
tion of p21-luciferase reporter was considerably enhanced when ERα was 
knocked down by siRNA, whereas transcription was severely reduced when 
Erα knockdown did not result in increased FAS/APO-1/CD95 (1) gene, was increased in response to ionizing 
radiation; however, unlike p53 alone or p53 and ERα knocked down by siRNA, or p53 and ERα 
knockdown did not result in increased p21 transcription indicating that ERα 
mediated apoptosis. Future experiments should address this issue. 

In conclusion, we have uncovered a novel mechanism by which ERα, generally 
up-regulated in breast cancer (6, 8), suppresses p53 function. Increasing evidence 
indicates that p53 dysfunction is an important event in breast cancer (27, 28). It is possible 
that interaction between p53 and ERα resulting in their reciprocal regu-
lation plays an important role in regulating normal breast epithelial cell prolifera-
tion, and aberration of this control may lead to breast cancer onset and progres-
sion.

Future studies should reveal the mechanisms and consequences of the 
interaction under different physiological and pathological contexts. Furthermore, 
whether ERβ (29) plays any role in this regulatory mechanism remains to be elu-
cidated. Besides unraveling a novel cellular pathway, these results have important 
clinical implications. ERα binding to p53 resulting in functional inactivation could 
be one of the reasons for the inability of wild type ERα to restrain the tumor growth 
and metastasis in ERα-positive breast cancer. Our observations open the oppor-
tunity for clinical evaluation of the importance of ERα-p53 interaction in tumors 
containing wild type ERα and p53. ERα and p53 status of tumors may be useful in 
developing a rational basis for combination therapy with ERα modulators and 
radiation. Furthermore, identification of agents that can significantly disrupt pro-
tein-protein interaction (30) between p53 and ERα might have cancer preventive 
and therapeutic potential.

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FIGURE 4. ERα antagonizes p53 during cell proliferation. A, MCF7 cells were trans-
fected with NS siRNA, ERα siRNA (50 nM), or p53 siRNA (20 nM). Cells were collected 24 h 
post-transfection and subjected to PI staining and fluorescence-activated cell sorter 
analysis. B, 24 or 48 h after transfection with siRNAs, cells were incubated with BrdUrd for 
45 min and processed for flow cytometry. The percentage of S phase cells was deter-
mained based on quantitation of the R4 region in the top panel. Total DNA was stained 
with 7-aminoactinomycin D (7-AAD).