Estrogen Receptor Protects p53 from Deactivation by Human Double Minute-2

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Abstract

We and others have demonstrated that estrogen receptor α (ERα) and p53, two important regulatory proteins in breast cancer, bind to each other. In this report, using the glutathione S-transferase pull-down methodology, we show the ligand-independent interaction of ERα with the NH2-terminal region of p53, a region known to bind the p300 and human double minute-2 (hdm2) regulatory factors. Furthermore, we have demonstrated that ERα is capable of binding hdm2 directly. The interaction of ERα and p53 does not interfere with the binding between p53 and hdm2; rather, these proteins form a ternary complex. The effect of ERα on the p53-hdm2 regulatory loop has been examined. Our results indicate that ERα protects p53 from being deactivated by hdm2. It is evident from these investigations that the ligand-independent protection of p53 by ERα is a novel role for this protein in addition to its classic regulatory function as a ligand-inducible transcription factor. This study also describes a new mechanism of cellular regulation of p53 activity.

Introduction

ERα is a ligand-inducible transcription factor that activates the transcription of genes that contain an estrogen response element in their promoter region. Genes that have been shown to be estrogen responsive include pS2 (1), cathepsin D (1), vitellogenin (2), c-fos (3, 4), c-jun (5), bel-2 (5), adenosine deaminase (6), transforming growth factor-α (7), and tissue plasminogen activator (8). Uterine tissue displays an additional tissue-specific function of ERα that does not require a direct interaction between receptor and DNA (9). Rather, ERα activates the regulatory factor, activator protein-1, by direct protein/protein interaction of the receptor complexed with agonist or positive (ERα) activity is blocked by the antagonist tamoxifen, certain ERα ligands, and hdm2 proteins were made by coupled transcription/translation (TNT T7 Quick Coupled Transcription-Translation kits; Promega, Madison, WI). The pSG5-hERα (HEGO) and the pCR3.1-hdm2 (generated by recloning hdm2 into the pGEX-6p-x vector) plasmids by cloning the full-length or truncated forms of the p53 cDNA into the pGEX-6p-x vector (Pharmacia Biotech, Piscataway, NJ).

Primers and PCR. The following primers were synthesized by Integrated DNA Technologies, Inc. (Corvalle, IN) and were used in PCR to synthesize DNA fragments for fusion proteins. P53_C_EcoRI, TGAATTCAGTCTGAGTCAGGCCCT (note: p53 aa393 reverse); p53_B_BamHI, TAGGATCCCATGGAAGAGGCGCAGT (note: p53 aal forward); P53MR, AAACTCAGGCTCCCCTTCTTCCGG (note: p53 aa290 reverse); P53MF, TTTGATCTACAGGGGCAACCTAGGT (note: p53 aai100 forward); ERα 5′ end, TATAACGCGTAATTCCGGACAGGACCACAT (note: ERα 5′ end nucleotides were used to generate the EcoRI linker); and hERα-C_Xhol, ATACCTGAGCTCTCAAGACTGGCGAGGA (note: ERα 3′ end plus the Xhol linker). The Expiland High Fidelity PCR kit (Boehringer Mannheim) was used for PCR (conditions: 95°C, 15 s; 61°C, 25 s followed by 72°C, 1 min and 45 s for 32 cycles).

In Vitro Translation and GST Pull-Down Assays. [35S]Met-labeled ERα and hdm2 proteins were made by coupled transcription/translation (TNT T7 Quick Coupled Transcription-Translation kits; Promega, Madison, WI). The pSG5-hERα (HEGO) and the pCR3.1-hdm2 (generated by recloning hdm2 cDNA into the pCR3.1 vector) plasmid DNAs were used as templates. The GST-p53wt (aminocids 1–393), GST-p53-wt (aminocids 103–295), and GST-p53-C (aminocids 103–393) fusion proteins were prepared according to the protocols that accompanied kits purchased from Pharmacia Biotech (Piscataway, NJ). Briefly, BL21(DE3)pLysS cells carrying the fusion protein plasmids were induced for 1.5 h by a 0.2 mM isopropyl-β-D-thiogalactopyranoside, lyzed, and analyzed for GST activity by the 1-chloro-2,4-dinitrobenzene assay (Pharmacia Biotech). Twenty μg of Sepharose 4B-GSH conjugated fusion protein were allowed to incubate with 5 μl of in vitro translated protein in 500 μl of HEPES buffer [50 mM KCl, 20 mM HEPES (pH 7.9), 2 mM EDTA, 0.1% NP40, 5% glycerol, 0.5% nonfat dry milk, and 5 mM DTT] at 4°C overnight or 37°C for cancer cells (16) were both growth inhibited when stably transfected with ERα.

The recent discovery of a physical interaction between p53 and ERα (17) and other steroid receptors (18, 19) suggested that the ERα-p53 complex might possess a function in cellular biology. To this end, p53 has been shown to take part in interactions with various other essential proteins such as p300 (20), mdm2 (21, 22), SV40TAg (22), ARF-14 (23), BRCA1 (24), and BRCA2 (25). Here, we report a further examination of the ERα-p53 interaction and describe a novel role for ERα in the protection of p53 from deactivation by the hdm2.

Materials and Methods

Plasmids. pSG5-hERα (HEGO) was a gift from Dr. Pierre Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, CNRS/INSERM/ULP/College de France, BP 163, CU de Strasbourg, France). pBS-mdm2 was a gift from Dr. Donna George (University of Pennsylvania School of Medicine, Philadelphia, PA). The hdm2 cDNA insert of this plasmid was used to generate the pCR3.1-hdm2 (which has a CMV and a T7 promoter and is suitable for both eukaryotic expression and in vitro transcription/translation). pWWP-Luc (26) and pC53-SN3 (containing the p53wt cDNA insert) were gifts from Dr. Bert Vogelstein (The Johns Hopkins Oncology Center, Baltimore, MD 21231). The human p53wt insert of this plasmid was used to generate the pGST-p53wt(aa1–393), pGST-p53-N(aa1–295), pGST-p53-M(aa103–295), and pGST-p53-C(aa103–393) plasmids by cloning the full-length or truncated forms of the p53 cDNA into the pGEX-6p-x vector (Pharmacia Biotech, Piscataway, NJ).

Received 1/6/00; accepted 2/17/00.

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These investigations were supported in part by NIH Grants DK 54837 and CA 68655.

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2 The abbreviations used are: ERα, estrogen receptor α; GST, glutathione S-transferase; hdm2, human double minute-2 oncogene; E2, 17β-estradiol; wt, wild type; HRP, horseradish peroxidase; Luc, luciferase; CMV, cytomegalovirus; CHO, Chinese hamster ovary; GSH, reduced glutathione.
1. Unbound proteins were removed with four washes of 500 μl of HEPES buffer. Bound proteins were eluted by boiling in 30 μl of 1X SDS loading buffer and resolved by SDS-PAGE. The gels were then fixed for 30 min in the protein fixing solution, equilibrated in Amplify fluorographic reagent (Amer- sham Life Science, Inc., Arlington Heights, IL) for 20 min, dried, and visualized by autoradiography.

Cell Maintenance, Transfection, and Luciferase Assays. HeLa cells were maintained in DME/F-12 media supplemented with 10% fetal calf serum plus 0.5% gentamicin and subcultured once per week. For transfections, the cells were passaged into growth medium containing heat-inactivated, dextran-coated, charcoal-stripped serum (8). Transient transfections were performed using the Superfect reagent (Qiagen, Inc., Chatsworth, CA) according to the manufacturer’s instructions. HeLa cells (50–70% confluent) were co-transfected overnight with 2 μg of pWLPW-Luc and 1.0 μg of pCR3.1-hm2, 1.0 μg of pAlt-p53, and 0.25 μg of pCMV5-βERa as indicated. Empty expression vectors (pCR3.1 and pCMV5) were used to reach a final DNA concentration of 4.25 μg in each sample (1.5 μg of pCH0110; β-galactosidase expression plasmid was added as internal control). The transfected cells were then incubated with or without ligands for 24 h prior to harvesting. Lysates were normalized for protein concentration or β-galactosidase activity and assayed for luciferase activity, using the TD 20/20 Luminometer (Turner Designs, Sunnyvale, CA) for quantification. The β-galactosidase activity and the luciferase activity were assayed using kits provided by the Promega Corp. (Madison, WI).

DNA Recombination and Cloning. The vector DNA and the insert fragment (1:3 molar ratio; 100 ng), which has been digested by appropriate enzymes, was added to T4 DNA ligase in 10 μl of buffered solution. The ligation reaction was allowed to proceed at 16°C overnight. Ligates were then used to transform 100 μl of competent JM109 cells for 30 min on ice. The transformed cells underwent heat shock for 40 s at 42°C. Super Optimal Catabolite medium was added, and the cells were incubated at 37°C for 1 h with shaking, after which the cells were plated onto agar with the selective antibiotic. After 18 h, the colonies were isolated and inoculated into tubes containing 3 ml of liquid broth containing the selective antibiotic. Thereafter, plasmid DNAs were isolated, and restriction digestion analysis was carried out to identify the correct clones.

Western Blotting. MCF-10A and 139B6 cells were grown in medium as reported previously (15). Cells (70–80% confluency) were removed from flasks with a rubber policeman and washed by centrifugation three times with cold PBS, then lysed in RIPA buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS containing an additional 100 μl of 10 mg/ml PMSF, and 1 tablet of the complete mini protease inhibitors; Boehringer Mannheim/Roche Molecular Biochemicals, Indianapolis, IN). Cellular lysates were measured for their protein concentrations by the BCA (Biomarker; Pierce, Rockford, IL), and aliquots were added to lanes on SDS-PAGE. After electrophoresis, the gels were sandwiched, and sample was transferred to a nitrocellulose membrane. After blocking the membrane with 5% dry milk in PBS, the first antibody (1:2500 dilution) against the target protein was allowed to interact, followed by the secondary antibody (1:3000 dilution). Samples were then visualized by standard ECL method (Amersham Life Science, Inc., Arlington Heights, IL). The antibodies used were: H222, rat monoclonal antibody against human ERα, supplied by Abbott Laboratories (Abbott Park, IL); actin (I-19), goat polyclonal antibody against human actin; p53(Bp53-12), mouse monoclonal antibody against human p53 (both wt and mutant); HDM2(SMP14), mouse monoclonal antibody against hdm2; HRP-conjugated goat antirabbit IgG; HRP-conjugated goat antimouse IgG; and HRP-conjugated donkey antigoat IgG (all purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

**Results**

ERα binds to the NH2-terminal of p53. The interaction between ERα and p53 (17) was confirmed in a GST pull-down assay in which in vitro translated ERα was incubated with Sephrose 4B-GSH-conjugated GST-p53wt (Fig. 1, a and b). The binding of ERα to p53 is not affected by the presence in the incubation of the ERα agonists, E2 and genistein, or antagonists, 4-hydroxytamoxifen and ICI164,384.

Data from experiments designed to determine the domains on p53 that bind with ERα are shown in Fig. 1c. Three truncated GST-p53 fusion proteins were constructed, each composed of specific functional domains of this regulatory protein. GST-p53-N contained the NH2-terminal 295 amino acids, which included the NH2-terminal transactivation domain and the sequence-specific DNA binding domain and the sequence-specific DNA binding domain. GST-p53-M was made up of amino acids 103–295, which encompassed the sequence-specific DNA binding domain, and GST-p53-C consisted of the amino acids 103–393, which encompassed the sequence-specific DNA binding domain and the COOH-terminal regulatory domain. Each incubation contained the same amount of radioactive ERα, an equal amount of the truncated fusion proteins (determined by the 1-chloro-2,4-dinitro-benzene assay for GST activity) and E2, 4- hydroxytamoxifen, or no ligand. Binding occurred when ERα was incubated with a fusion protein containing the NH2-terminal 102 amino acids of p53 (Fig. 1, c and d). Apparently, the protein-protein interaction involved some of the residues beyond amino acid 103, because GST-p53-M and GST-p53-C displayed a minor interaction with the receptor. Ligands had no effect on the binding.

Studies have shown that the p53 NH2-terminal is the binding region for both p300 (20) and hdm2 (21). The fact that p300 can bind to both ERα and p53 brings about two possible scenarios: (a) p300 might bring ERα and p53 together; and (b) ERα may compete with p53 for binding to p300. Results from experiments in this laboratory (27), which demonstrated that p53 does not compete with p300 in the
suppression of ERα activities, suggest that p300 does not play a role in the ERα-p53 interaction.

**ERα, p53, and hdm2 Form a Ternary Complex and ERα Can Bind to hdm2 Directly.** Interestingly, when increasing amounts of [35S]Met-labeled ERα (0.5–10 μl, with 10⁻⁷ M 4-hydroxytamoxifen) were added to the tubes containing hdm2 and the GST-p53 fusion proteins, there was a 3.3-fold rise in the amount of hdm2 being pulled down by the GST-p53-N fusion protein (above that pulled-down in the absence of ERα; Fig. 2a). The amount of ERα in this complex increased accordingly. A similar observation was made when the GST-hdm2 fusion protein was used to pull-down in vitro translated ERα and p53 proteins. In this case, increasing input of ERα did not reduce the amount of p53 pulled-down by the GST-hdm2 fusion protein (Fig. 2b). These data suggest that ERα, p53, and hdm2 form a ternary complex.

Support for the formation of a ternary complex was gained from experiments carried out to examine the binding of ERα and hdm2. In these experiments, GST fusion proteins of the full-length and a truncated ERα were incubated with in vitro translated [35S]Met-labeled hdm2 protein in the absence or presence of E2 (Fig. 3). These experiments showed that both the full-length and the AB domain-deleted ERα-GST fusion proteins were capable of direct physical binding to hdm2 in the presence and in the absence of ligand.

**ERα Protects p53 from Being Deactivated by hdm2.** The formation of a ternary complex composed of ERα, p53, and hdm2 suggests a role for the receptor in the p53-hdm2 regulatory loop (28). Possibly ERα influences the ability of hdm2 to down-regulate the transactivity of p53 (28). To test this hypothesis, HeLa cells were transiently cotransfected with the p53 responsive reporter pWWP-Luc (26) and expression plasmids for human hdm2 (pCR3.1-hdm2), p53 (pAlt-p53), and/or ERα (pCMV5-hERα). As shown previously (26), the luciferase activity increased when p53 was cotransfected with the pWWP-Luc plasmid (Fig. 4, groups 1 and 2). When hdm2 was added, p53 became ineffective in inducing the activity of this reporter gene (Fig. 4, groups 3 and 4). Interestingly, coexpression of ERα restored the p53-driven WWP-Luc activity (Fig. 4, group 5). Alone, or in the presence of hdm2, ERα had no effect on the activity of WWP-Luc. In a separate experiment, the restoration of p53-stimulated luciferase activity was shown to be dependent on the amount of pCMV5-hERα transfected (range, 0.05–0.4 μg; data not shown). The down-regulation of p53 by hdm2 was completely reversed by a transfected level of ERα plasmid between 0.20 and 0.40 μg. These results suggest that ERα protects p53 from being deactivated by hdm2.

**Expression of ERα Leads to Elevated p53 and hdm2 Protein Levels.** p53 has been shown to augment the expression of hdm2 (28). In turn, hdm2 also controls the level of p53 in cells via the ubiquitin-proteasome pathway (21), which decreases the level of this tumor suppressor. The presence of ERα in cells would be predicted to protect p53 from hdm2-targeted degradation and lead to an increase in the level of p53. According to this line of reasoning, a cellular increase in p53 should enhance the expression of hdm2. The MCF-10A human breast epithelial cell line normally does not contain ERα (15). Transfection of ERα into these cells created an ERα+ stable cell line, 139B6 (15). A Western blot of the proteins in these transfected cells demonstrated that the presence of ERα is accompanied by increasing levels of both p53 and hdm2 proteins above those detected in the parental ER− cell line (Fig. 5). Interestingly, the 139B6 cells have a longer doubling time than the vector-transfected MCF-10A cell line (15), possibly a result of ERα protection of the p53 that acts to lengthen the G1 phase of the cell cycle.
**Discussion**

The tumor suppressor p53 and the oncogene hdm2 display an essential interplay in regulating cellular proliferation and apoptosis. It has been demonstrated that p53 up-regulates the expression of hdm2 via a stimulation of its mRNA transcription (28). On the other hand, hdm2 suppresses the p53 transactivity by interfering with the interaction between p53 and the basic transcriptional machinery (29). hdm2 also controls the level of this tumor suppressor protein through the ubiquitin-proteasome pathway (21). Results from this investigation point toward a role for ERα in this essential regulatory pathway (Fig. 6).

Using the GST pull-down assay, we have demonstrated that ERα is capable of binding to the NH2 terminus of the p53 protein (Fig. 1). This interaction is unaffected by the presence of ligand. As a further indication of the interworking of these proteins, we have shown that ERα positively influences the binding of hdm2 and p53 (Fig. 2), although both factors bind to the NH2 terminus of p53. The ability of ERα to protect p53 from functional deactivation by hdm2, as demonstrated in a Luc reporter assay (Fig. 4), suggests that the ternary complex formed by ERα, p53, and hdm2 may have an important functional role. Our findings that p53 (and thus hdm2) levels are increased in ERα-transfected immortalized breast epithelial cells, compared with those of the parental cell line, provide additional support for this conclusion (Fig. 5).

The relationship between ERα and hdm2 has been observed previously. Sheikh et al. (30) reported that ER+ breast cancer cells have significantly higher (up to 30-fold) hdm2 mRNA levels than those of ER− breast cancer cells. In addition, MDA-MB-231 cells, which have been stably transfected with the ERα cDNA, produced a 3-fold increase in the hdm2 mRNA levels (30). In the absence of an ERE in the promoter of the hdm2 gene, it appears that the observed stimulatory effect of ERα on the hdm2 is carried out via the elevated p53 transcriptional activity (hdm2 is positively regulated by p53; Figs. 5 and 6).

Other than p53, three important regulatory proteins [p300 (31), p19ARF (32) and Rb (33)] have been reported to bind to hdm2. All of these proteins compromised the ability of hdm2 to degrade p53. Furthermore, the protective effects of p19ARF had been shown to occur via its ability to inhibit the ligase activity of hdm2. To this end, our results have demonstrated that ERα also protects p53 from being deactivated by hdm2 (Fig. 4). ERα is capable of binding hdm2 directly (Fig. 3), as well as elevating levels of p53 and hdm2 in ERα stably transfected MCF10A cells (Fig. 5). This suggests that ERα might suppress the ubiquitin ligase activity of hdm2.

In normal breast tissues under nonlactating conditions, ERα is present only in ~7% of the epithelial cells (34). This level may increase or decrease in breast tumor tissue. Often, ERα expression is lost during the progression of breast cancer. Furthermore, there is an inverse correlation between ERα expression and malignant progression in mammary neoplasia (34). Because ERα expression is up-regulated in tissues that are rapidly dividing, such as the uterine endometrium during the proliferative phase, it is conceivable that the
accompanying elevated p53 activity plays a role in the prevention of rapidly growing tissues from becoming transformed.

Other investigators (13, 14, 16) have observed increased doubling times after they introduced ERα into ER− cells. This role played by ERα is very similar to the role of the p14ARF tumor suppressor (23), which binds to both p53 and hdm2 and protects p53 from being down-regulated by hdm2. Over the past decade, the cytotoxicity resulting from ERα overexpression (13) has been observed repeatedly (14–16, 30). Nevertheless, this observation remains unexplained. In one classic example, Kushner et al. (13) overexpressed ERα in CHO cells and found that even trace amounts of E2 (or tamoxifen) were lethal to these stably transfected cells. On the basis of the present information, the enhanced ERα-mediated protection of the wt53 may have resulted in the death of these cells. Just as the double knock-out of the mdm2 gene is lethal to the embryonic development (35), overprotection of p53 from hdm2 deactivation can lead to growth retardation or even lethality. It appears that elevated p53 is responsible for the increased hdm2 levels, and it would follow that the elevated protection of p53 may explain the toxicity that is associated with ERα overexpression.

These investigations have resulted in the novel finding that ERα functions to protect p53 from hdm2-induced deactivation. This role of the unliganded receptor is quite unlike the classic function of ERα which, once bound to E2, regulates target genes containing the specific response element in their promoters. Such genes take part in mitogenesis as well as differentiation (1–8).

Acknowledgments

The authors are grateful to Drs. B. Vogelstein, P. Chambon, D. George, and R. H. Goodman for providing plasmids used in these experiments.

References

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