Differential regulation of estrogen receptor alpha turnover and transactivation by Mdm2 and stress-inducing agents.

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Mdm2 and stress-inducing agents regulate estrogen receptor α turn-over and transactivation.

Running title: Stress and estrogen signaling

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ABSTRACT

In mammalian cells, the level of estrogen receptor α (ERα) is rapidly decreased upon estrogen treatment and this regulation involves proteasome degradation. Using different approaches, we showed that the Mdm2 oncogenic ubiquitin-ligase directly interacted with ERα and is involved in the regulation of ERα turn-over. We showed that Mdm2 overexpression decreased ERα stability (both in the absence or presence of estrogens) and deletion of the ubiquitin-ligase domain abolished the negative regulation of ERα expression. Interestingly, our results indicated that ligand-dependent receptor turn-over was not required for efficient transactivation. Moreover, several lines of evidence suggested that Mdm2 interact with ERα in a ternary complex with p53. In MCF-7 human breast cancer cells, various p53-inducing agents (such as UV irradiation) or treatment with RITA (which inhibits the interaction of p53 with Mdm2) stabilized ERα and abolished its E2-dependent turn-over. Finally, in addition to this early positive effect on ERα stability, we showed that cellular stress inhibited ERα mRNA accumulation leading to a strong delayed inhibition of receptor expression. Altogether, our results indicate that ERα expression is complexly regulated by cellular stress, acting both on gene transcription and protein stability by targeting the Mdm2 protein.

INTRODUCTION

Estrogens are key regulators of cell differentiation and proliferation and these hormones play important roles in female reproduction physiology and tissue homeostasis. They exert their biological action *via* specific receptors (ERα and ERβ) which are members of a superfamily of hormone nuclear receptors, acting as gene regulatory transcription factors (43). Upon ligand binding, ERs regulate gene expression through the binding to their cognate estrogen response elements (ERE) or *via* protein-protein interactions with transcription factors such as AP-1 or Sp1. In the presence of hormone, ERs undergo a major conformational change allowing the recruitment of transcriptional cofactor complexes, which in turn engage the basal transcription machinery and/or act to locally modify chromatin structure and subsequently stimulate expression of estrogen-responsive genes.

Liganded-nuclear receptors recruit various types of enzymatic activities that participate in gene expression regulation (34). Previous studies have suggested that ubiquitin-conjugating enzymes or ubiquitin-protein ligases such as UbcH5/UbcH7 (40, 45), RPF1/RSP5 (20) or E6-AP (41) interact with members of the nuclear receptor superfamily and modulate their transactivation functions. Similarly, ATPase subunits of the proteasome complex such as TRIP1/SUG1 (27) or TBP1 (21) also bind nuclear receptors and modulate their functions.

More than 30 years ago, Jensen and collaborators have demonstrated that estradiol (E2) treatment significantly reduces ERα levels in the uterus of ovariectomized rats (23). More recently, several studies have shown that binding of E2 to ERα significantly decreases its stability. This shorter half-life in the presence of hormone appears to implicate the ubiquitin/proteasome pathway since ERα has been shown to be ubiquitinated (58) and the ligand-dependent down-regulation is blocked by proteasome inhibitors (1, 12, 40). Interestingly,

although partial antiestrogens such as tamoxifene also increase ERα accumulation, pure antihormones such as the ICI182,780 compound strongly decrease receptor stability (10, 58). Several reports have suggested that the proteasome may control not only ERα protein levels but also hormone-dependent transcription (32). This effect is associated with the immobilization of ERα on the nuclear matrix as determined by fluorescence recovery after photobleaching (FRAP) (54). Although several candidates have been proposed to account for the E2-dependent regulation of ERα expression, the exact molecular mechanisms still remain unraveled.

The Mdm2 oncogene is overexpressed in a wide variety of human cancers (38) and its role in tumorigenesis is linked to its ability to act as an E3 ubiquitin-ligase (18) which mediates the ubiquitination and proteasome-dependent degradation of several growth regulatory proteins including p53 (16, 37). Interestingly, it has been previously suggested that Mdm2 could directly interact with ERα (31, 49) and a positive effect of Mdm2 overexpression on ERα activity has also been reported (49).

In the present study, we show that the Mdm2 oncoprotein interacts with ERα and is involved in the ligand-dependent decrease of receptor stability. Our data indicate that Mdm2 regulates ERα expression as a ternary complex with p53 and, in support of this observation, we show that various stress-inducing agents (which stabilize p53) block E2-dependent regulation of ERα stability in MCF-7 human breast cancer cells. Finally, this study provides several lines of evidence showing that the E2-dependent turnover of the receptor is not necessary for ERE-mediated transactivation.

MATERIALS AND METHODS

Plasmids and reagents. The ERα expression vectors (wild-type and deletion mutants) were given by P. Chambon (IGBMC, Strasbourg, France). The GST-AF2wt and GST-AF2mut vectors (9), the plasmids encoding GST-C/D and ER-VP16 (56) and the ERE-βGlob-Luc and 17M5βGlob-Luc (5) reporter constructs were described elsewhere. The GST-p53 and the pcDNA3 plasmids encoding full-length p53 (26) were obtained from U. Hibner (IGMM, Montpellier). The pXJ-mdm2 vector was obtained from B. Wasylyk (IGBMC, Strasbourg, France) and the pcMI-Hdm2 contained the Mdm2 cDNA (BamHI/EcoRI fragment) subcloned into the pCMI1 vector (42). The pcMI-Hdm2ΔRING was obtained by inserting into the pCMI1 vector a PCR-amplified BamHI/EcoRI fragment corresponding to amino acids 1 to 435. The GST-mdm2 was constructed by cloning a XhoI/Not1 fragment of the human Mdm2 cDNA into the pGEX4T3 vector (Amersham Biosciences, Saclay, France). The Gal-Mdm2 was generated by introducing a blunted NcoI/BglII fragment from pXJMdm2 into the SmaI site of the pSG424 plasmid (48). The RITA compound (NSC652287) was obtained from Dr R.J. Schultz (NIH, DTP, DSCB, Rockville, MD, USA).

Cell culture. Monolayer cell cultures (MCF-7, HeLa, U20S) were grown respectively in Ham's F-12/Dulbecco's modified Eagle's medium (1:1) (F12/DMEM) or DMEM alone supplemented with 10% fetal calf serum (FCS) (Invitrogen, Cergy-Pontoise, France) and antibiotics. Before hormonal treatments, cells were stripped of endogenous steroids by passage in medium without phenol red containing 3% charcoal-stripped FCS (FCS/DCC). Control cells were grown under the same conditions and complemented with vehicle alone (ethanol). When indicated, cells were irradiated using a Stratalinker UV crosslinker (Model 1800) from Stratagene (Montigny-le-Bretonneux, France). The MELN cell line derived from MCF-7 cells

stably transfected with the ERE-βGlob-Luc-SVneo plasmid (4). Mouse embryo fibroblasts null for both p53 and Mdm2 (MEFdKO) (35) were obtained from G. Lozano (Houston, Texas) and cultured in F12/DMEM.

Transient transfection and luciferase assays. HeLa cells and MEFdKO were plated in 6-well plates (10^5 cells per well) 24h prior to DNA transfection (4µg of total DNA) by the calcium phosphate method using CMV-βGal expression vector as an internal control. For measurement of E2-dependent transactivation and protein accumulation in MELN cells, cells were plated in 6-well plates (5.10^5 cells per well) and were treated in parallel for luciferase assay and Western-blot analysis. To quantify luciferase activity, cells were lysed at 4°C for 10min in 400µl of lysis buffer (25mM Tris pH 7.8, 2mM EDTA, 10% glycerol, 1% Triton X-100). Luciferase activity was measured on 100µl of supernatant aliquots by integrating photons emitted during 2s after injection of 100µl of luciferase detection solution using a luminometer (LB 960 Berthold). Luciferase values from transient transfection were normalized by the β-galactosidase activities as described (8) and all data were expressed as mean $\pm$ SD.

Western-blot analysis. Whole cells extracts were prepared in high-salt lysis buffer (HSB) containing 500mM NaCl, 50mM Tris pH8, 1% Nonidet P-40, 1mM dithiothreitol and proteases inhibitors (Roche Diagnostics, Meylan, France). Insoluble material was removed by centrifugation. Proteins were quantified using the Bradford assay (Bio-Rad Laboratories, Marnes, France) and 30µg were usually loaded on SDS-PAGE and transferred to PVDF membrane. Blots were saturated in TBST buffer (50mM Tris pH7.5, 150mM NaCl, 0.1% tween 20 (v/v), 5% nonfat dehydrated milk (w/v)), incubated with specific primary antibodies for ERα (sc-543 from Tebu, Le Perray, France), p53 (sc-126 from Tebu, Le Perray, France) Mdm2 (clone 2A10, gift from J. Piette, IGMM, Montpellier) or actin (Sigma) and probed with the appropriate

secondary antibody (Sigma). Detection was done using the Chemiluminescence Reagent Plus kit (Perkin Elmer Life Science, Courtaboeuf, France).

Coimmunoprecipitation experiments. Transiently transfected cells were washed in PBS, centrifuged and frozen at -80°C. After thawing, cells were lysed and sonicated in 50mM Tris pH8, 5mM EDTA, 150mM NaCl, 0,5% NP40 supplemented with 1mM PMSF. Preclearing of cell extracts was realized by incubation with protein A sepharose for 10 mn at 4°C. After centrifugation, precipitation of Mdm2 was carried out for 2 h at 4°C using the anti-Mdm2 antibody (clone 4B11, gift from J. Piette, IGMM, Montpellier) in a final volume of 500µl. Beads were washed 3 times in 0.5 ml of SNNTE buffer (50mM Tris pH7.4, 500mM NaCl, 5mM EDTA, 5% sucrose, 1% NP40) and once in RIPA buffer (50mM Tris pH7.4, 150mM NaCl, 1% Triton X100, 0.1% SDS, 1% Na deoxycholate). Finally, the beads were resuspended in 30µl of loading sample buffer and immunoprecipitated proteins were analyzed by SDS-PAGE.

GST-pull down assays. *In vitro* translation and GST pull-down assays were performed as previously described (8). Briefly, ^{35}S-labeled proteins were cell-free-synthesized using the TNT lysate system (Promega) and incubated with purified GST fusion proteins overnight at 4°C in NETN buffer containing 0.5% Nonidet P-40, 1mM EDTA, 20mM Tris pH8, 100mM NaCl, 10mM DTT and proteases inhibitors cocktail (Roche Diagnostics, Meylan, France). Protein interactions were analyzed by SDS-PAGE followed by quantification using a Phosphorimager (Fujix BAS1000). Gels were stained with Coomassie Brilliant Blue (BioRad) to visualize the GST fusion proteins present in each track.

Apoptosis assay. MCF-7 cells were plated in 6-well plates (50 000 cells/well) and treated or not with MG132 (4µM), actinomycin D (4nM) or UV irradiated. Apoptosis was quantified 24h later using the Cell Death Detection ELISA (Roche Molecular Biochemicals, Meylan, France),

according to the manufacturer's conditions. Values from absorbance measurements at 405nm were corrected using DNA quantification in separate wells treated in parallel.

RNA extraction and quantitative PCR. Total RNA was extracted using RNeasy kit (Qiagen, Courtaboeuf, France) according to the manufacturer's conditions. For RT-PCR, 1.5µg of total RNA was subjected to reverse transcription using the Omniscript Reverse Transcriptase kit (Qiagen, Coutaboeuf, France). Real-time PCR quantification was then performed using a SYBR Green technology (Light Cycler Roche). For each sample, ERα mRNA levels were corrected for HPRT mRNA levels used as a reference gene and normalized to a calibrator sample. The primers for ERα and HPRT mRNAs have been described elsewhere (11).

RESULTS

ERα is involved in a ternary complex with p53 and Mdm2. In order to characterize protein-protein interactions between Mdm2 and ERα, we first performed GST-pull down experiments. As shown in Figure 1A (left panel), we observed a ligand-independent interaction of *in vitro* translated ERα with Mdm2 expressed as a fusion protein with GST, whereas no detectable interaction was obtained with GST alone (data not shown).The binding of Mdm2 to ERα was mediated by the ligand binding domain (LBD) of the receptor, as shown by the recruitment of *in vitro* expressed Mdm2 with the GST-LBD protein, which was also unaffected by E2 or by the partial antiestrogen 4-hydroxytamoxifen (right panel). Moreover, the use of a GST-LBD mutated in the conserved AF2 activation domain indicated that this interaction did not require an intact AF2 transactivation domain (data not shown). The use of ERα deletion mutants (shown in Figure 1B, left panel) confirmed that both the DBD (HE11 mutant) and the N-terminal region (HE19 mutant) were not necessary for the binding to Mdm2 (Figure 1B, right panel). By contrast, our data confirmed that the C-terminal LBD of ERα was required for the *in vitro* interaction since the HE15 mutant poorly associated with GST-Mdm2. Altogether, these results demonstrated a direct ligand-independent interaction of ERα with Mdm2.

In order to confirm that these interactions also occurred in intact cells, we performed two types of experiments. We first set up coimmunoprecipitation experiments using an anti-Mdm2 antibody to precipitate overexpressed Mdm2 and we analyzed co-precipitated proteins for the presence of transfected ERα. As illustrated in Figure 1C, endogenous Mdm2 was efficiently precipitated by the anti-Mdm2 specific antibody (clone 4B11) and ERα was associated with immunoprecipitated Mdm2, as determined by probing the Western blot with a polyclonal antibody specific for the receptor (lanes 1 and 2). Interestingly, when the experiment was carried

out in MEFdKO embryonic fibroblasts (39) derived from p53-/- and Mdm2-/- mice (lanes 3 and 4), ERα was not coprecipitated with Mdm2 suggesting that p53 could be required for the interaction between ERα and Mdm2. Such a hypothesis was supported by the fact that p53 also directly interacted with ERα in a ligand-independent manner (31). Interestingly, in support of this study, we found that the p53/ERα interaction domain encompassed the central region of the receptor (GST-C/D) *i.e.* different from that required for Mdm2 binding (Figure 1D).

The hypothesis that ERα could be engaged in a ternary complex with p53 and Mdm2 was confirmed using a modified mammalian two-hybrid system (Figure 1E). An expression plasmid coding for Mdm2 fused to the Gal4 DBD (Gal-Mdm2) was cotransfected in MCF-7 cells together with a Gal4 responsive reporter plasmid and an expression vector coding for ERα fused to the VP16 activation domain or for VP16 alone. In our experimental conditions, a slight but significant increase in luciferase activity (more than 2-fold) was obtained when ER-VP16 was coexpressed with Gal-Mdm2 (as compared to the activity obtained with VP16 alone). Interestingly, when a p53 expression plasmid was cotransfected with Gal-Mdm2 and ER-VP16, we observed a significant increase in luciferase activity (more than 10-fold) suggesting that p53 may indeed stabilize the interaction between ERα and Mdm2. Altogether, these results suggest that ERα, p53 and Mdm2 coexist within the same protein complex in intact cells.

p53 and Mdm2 are required for ligand-dependent ERα turn-over. In order to evaluate the role of p53 and Mdm2 in ligand-dependent down-regulation of ERα, we first used the MEFdKO model (39). Interestingly, in this p53/Mdm2-/- background, estrogen treatment did not decrease ERα accumulation (as observed in parallel in p53/Mdm2+/+ wild-type MEFs) but instead slightly increased receptor levels (Figure 2A). The same effect was observed whatever the

concentration of ERα expression vector used to transiently transfect these p53/Mdm2 -/- cells (data not shown). To demonstrate that the expression of p53 and Mdm2 was important for the E2-dependent inhibition of ERα accumulation, we transiently transfected increasing concentrations of expression vectors for both p53 and Mdm2 together with the expression vector for ERα in p53/Mdm2-/- MEFs (Figure 2B). The positive effect of E2 on ERα accumulation that we observed in control p53/Mdm2-/- cells was progressively diminished and, at the highest concentration of expression vectors for p53 and Mdm2, we restored the negative hormonal regulation.

Since previous studies proposed that the E2-dependent decrease of ERα accumulation was required for transcriptional activity of the receptor (32), we measured in parallel, the ability of the transiently transfected ERα to increase the transcription of a reporter gene in MEFs expressing or not p53/Mdm2. As shown in Figure 2C, we found that ERα strongly activated transcription in conditions where E2 up-regulated its accumulation. Very interestingly, we found that overexpression of p53/Mdm2 only slightly modify ERα transactivation although it completely inversed the effect of E2 on its accumulation.

Finally, to emphasize the importance of Mdm2 in ligand-dependant turn-over of ERα, we measured the effect of Mdm2 overexpression on the apparent stability of the ERα protein. This experiment was performed in E2-treated HeLa cells (which are p53 positive cells), by chase experiments using cycloheximide as a protein synthesis inhibitor. As shown in Figure 2D, we noticed a significant decrease in ERα apparent stability, thus emphasizing the role of Mdm2 in the post-translational regulation of receptor expression. Altogether these data suggest that i)

Mdm2 is involved in E2-dependent turn-over of ERα ii) a negative effect of E2 on ERα expression is not required for efficient transactivation.

Mdm2 regulates ligand-independent expression of ERα. Since some of the data shown in Figure 1 suggested that Mdm2 could also bind ERα in an E2-independent manner, we investigated the effect of Mdm2 overexpression on ERα accumulation in the absence of ligand. As shown in Figure 3A, when Mdm2 and ERα expression vectors were transiently cotransfected in HeLa cells, the accumulation of the receptor was strongly decreased. This effect depended on the amount of cotransfected expression vector for Mdm2 (Figure 3B). As expected, this decrease in the level of ERα accumulation correlated with a shorter apparent half-life of the protein (Figure 3C) and led to a lower ligand-independent ERα transactivation (Figure 3D). Interestingly, overexpression of Mdm2 in p53/Mdm2-/- MEFs also led to a 2- to 3-fold decrease in the basal level of ERα transactivation suggesting that this effect was p53 independent (data not shown).

We then tried to further decipher the molecular mechanisms involved in ERα degradation by Mdm2. We used a mutant of Mdm2 (Mdm2ΔRING) deleted in the C-terminal part of the protein which contains the RING domain required for its ubiquitin-ligase activity (19). As shown in Figure 4A, this mutant still interacted with ERα in GST-pull down experiment. Interestingly, overexpression of the Mdm2ΔRING mutant did not decrease ERα accumulation as compared with the effect of its wild-type counterpart (Figure 4B) suggesting that the E3 ubiquitin-ligase activity of Mdm2 is directly involved in ERα degradation.

Finally, in an attempt to compare the E2-dependent turn-over of the receptor to the ligand-independent degradation observed upon Mdm2 overexpression, we used a mutant of ERα

deleted in the central DNA-binding domain involved in the binding of p53 (see Figure 1B and D). This ERα-ΔDBD mutant (HE11) was no longer degraded upon E2 stimulation (Figure 4C, right panel lane 5) in support with the role of this domain in recruiting p53 and consistent with the formation of a ternary complex between the receptor, p53 and Mdm2 (Figure 1). By contrast, the ligand-independent increase in ERα turn-over observed upon Mdm2 overexpression was comparable for the wild-type protein and the DBD-deleted mutant (Figure 4C, lane 3 for ERα WT and lane 6 for HE11). Altogether, these data support the hypothesis that different mechanisms might be involved in the regulation of ERα turn-over upon E2 treatment or Mdm2 overexpression.

Stress-inducing agents block ligand-dependent turn-over of ERα. Previous studies reported that, in MCF-7 human breast cancer cells, the E2-dependent decrease of ERα accumulation was abolished by the MG132 proteasome inhibitor (1, 12, 40). Results shown in Figure 5A confirmed that MG132 was able to reverse the effect of various ligands such as E2, estrone (E1) or estriol (E3) on ERα levels in MCF-7 cells. Interestingly, proteasome blockade is a cellular stress which increased accumulation of p53 (Figure 5A) and as a consequence, induced apoptosis, as shown by quantification of cytoplasmic nucleosomes (Figure 5B).

Based on our results concerning the role of Mdm2 in the regulation of ERα turn-over, we thought that the effect of MG132 could be linked (at least in part) to its ability to dissociate Mdm2 from p53 and/or ERα. We therefore analyzed the effect of other stress-inducing agents (genotoxic or non-genotoxic) for their ability to regulate E2-dependent turn-over. As shown in Figure 5B, both UV irradiation and inhibition of transcription (actinomycin D treatment) increased significantly programmed cell death in MCF-7 cells. In all conditions, the accumulation of p53 was significantly increased to levels comparable to those obtained after

MG132 treatment and, very interestingly, both treatments that increase p53 levels concomitantly suppressed the hormone-dependent down-regulation of ERα (Figure 5C).

We then characterized the effect of UV irradiation on the regulation of ERα accumulation. We first showed that the blockade of E2-dependent turn-over after UV exposition was rapid since observed 4h after irradiation and still detectable 24h later (data not shown). We then investigated the effect of UV irradiation on the response to antiestrogens which have also been shown to regulate ERα expression. Indeed, as previously reported, incubation with the partial antagonist 4-hydroxy tamoxifen (OHT) increased the level of ERα whereas pure antiestrogens such as the ICI182,780 molecule (ICI) negatively regulated the accumulation of the receptor (58). As shown in Figure 5D, UV irradiation did not affect the stabilization of ERα upon OHTam treatment. Very interestingly, it did not antagonize the effect of ICI182,780 whereas it completely reversed the agonist-dependent decrease of ERα levels. This suggested that degradation of ERα by pure antiestrogens involved different mechanisms than those required for hormone-dependent degradation.

We then analyzed the effect of UV irradiation on endogenous ERα activity. To this aim, we used a cell line established in our laboratory (namely the MELN cells) which are MCF-7 cells stably transfected with an E2-regulated luciferase reporter gene allowing easy monitoring of endogenous ERα activity (5). MELN cells were treated with E2 at various time post irradiation by UV (*i.e.* when E2-dependent turn-over of ERα was no longer detectable). As shown in Figure 5E, the transcriptional activity of ERα was significantly increased both in the absence of ligand or in the presence of E2 or antiestrogens. In all conditions, the induction by E2 was detectable

confirming that E2-dependent turn-over was not a prerequisite for transactivation even on endogenous ERα.

Concomitant stabilization of ERα and p53 by stress-inducing agents. To confirm that UV irradiation modulated the stability of the ERα protein, we performed chase experiments with cycloheximide. As shown in Figure 6A and B, the stability of the receptor in the absence of *de novo* protein synthesis decreased upon E2 treatment and UV irradiation completely inhibited the hormone-dependent degradation. As expected, upon UV treatment, p53 appeared very stable and its accumulation was strongly increased (Figure 6A). Altogether, these data suggest that stress-inducing agents induce a co-stabilization of p53 and ERα in MCF-7 breast cancer cells. Our hypothesis was that disruption of the p53/Mdm2/ERα complex upon cellular stress was at the basis of the loss of E2-dependent turn-over of the receptor.

To further emphasize this point, we used the NSC652287 compound (also known as RITA which stands for *Reactivation of p53 and Induction of Tumor cell Apoptosis*), and recently described as an inhibitor of the p53/Mdm2 interaction (22). As shown in Figure 6C, treatment of MCF-7 cells with RITA at 1μM significantly abolished the ligand-dependent decrease in ERα accumulation. This effect was detectable 4h after the beginning of the treatment and very interestingly, it correlated again with the increase in p53 accumulation. Finally, when tested over the same period of time, we found no significant decrease in ERα transactivation in the presence of RITA (Figure 6D), thus supporting the data obtained after UV irradiation (Figure 5E). Altogether, these data suggest that dissociation of the p53/Mdm2/ERα complex upon cellular stress leads to a co-stabilisation of p53 and ERα proteins without significantly decreasing receptor transactivation.

Cellular stress inhibits ERα transcription. As a control of UV irradiation and RITA treatment, we also measured the levels of ERα mRNA by quantitative RT-PCR in order to check that no up-regulation of the mRNA was associated with the blockade of the E2-dependent decrease observed at the protein level. We were very surprised to find that both UV irradiation and RITA treatment strongly decreased ERα mRNA levels in MCF-7 cells both in the absence or presence of E2. Indeed, as shown in Figure 7A, the levels were lowered by 3- to 5-fold after 12h treatment with RITA in the presence or absence of E2, respectively.

We then verified that the accumulation of ERα protein was decreased after a long term treatment with stress-inducing agents. Western-blot analyses confirmed that both UV irradiation and RITA treatment (24h or 48h) strongly inhibited ERα expression (Figure 7B). As expected, this was associated with a significant decrease in ERα-dependent transactivation which was clearly observed 48h after UV irradiation or RITA treatment and already detectable for RITA after 24h of treatment (Figure 7C). Altogether, this suggested that stress-inducing agents exerted a dual effect on ERα expression and activity: over a short period of time, they stabilized the protein and increased its transactivation. By contrast, over a longer period, they decreased ERα expression at the transcriptional level, resulting in a strong repression of ERα-dependent transcriptional regulation.

DISCUSSION

Modulation of ERα levels is a critical parameter in determining the hormonal response of breast cancer cell proliferation. The control of ERα expression is under a complex regulation which takes place both at the transcriptional and post-transcriptional levels. Previous data suggested that ERα was down-regulated in the presence of E2 through a proteasome-dependent mechanism (1, 12, 40). In this study, we have investigated the mechanisms regulating the hormone-induced ERα turn-over and several lines of evidence indicate that Mdm2, an oncogenic ubiquitin-ligase, plays an important role in the regulation of ERα accumulation by E2.

First, using a modified two hybrid assay we show that Mdm2 interacted with ERα in a ternary complex with p53. The direct *in vitro* interaction between the receptor and both p53 and Mdm2 was also demonstrated using GST pull-down assays. Our data suggested that Mdm2 binding involved the LBD of ERα but did not require the ligand-dependent AF2 interface. Interestingly, p53 interacted with a different region of the receptor (*i.e*. the central region) thus supporting the formation of a ternary complex.

The use of p53/Mdm2-/- cells demonstrated that the two proteins were required for the E2-dependent down-regulation of ERα. Moreover, we showed that the C-terminal region of Mdm2 which encompassed the E3 activity was required for the degradation of ERα. Previous studies highlighted the role of the Mdm2 ubiquitin-ligase in the degradation of steroid hormone nuclear receptors. In the case of the glucocorticoid receptor (GR), Sengupta and Wasylyk showed that disruption of the p53/Mdm2 interaction prevented ubiquitination of GR and that the ligand-dependent trimeric complex between GR, p53 and Mdm2 enhanced proteasomal degradation of the receptor (51). Moreover, the E3 ligase activity of Mdm2 was also necessary for the ubiquitination and degradation of the androgen receptor (30).

From the data presented in our study, we propose that p53 and Mdm2 are involved in the ligand-dependent degradation of ERα. Interestingly, in support of our data, it has been recently demonstrated, by chromatin immunoprecipitation, that Mdm2 was recruited on the ERα-regulated pS2 promoter upon E2 stimulation (47, 57). Moreover, both Mdm2 and p53 expression levels are increased by E2 in MCF-7 breast cancer cells (24). This positive regulation could be of importance in the E2-dependent down-regulation of the receptor.

Obviously, other factors associated with p53 or Mdm2 could be involved in such a degradation complex. A strong candidate is the coactivator AIB1 which has been shown to interact with p53 (28) and to be required for the E2-dependent turn-over of ERα (53). The involvement of AIB1 could explain why the AF2 mutated ERα is no longer degraded upon hormone stimulation (V. Duong, unpublished data). Interestingly, in breast cancer, AIB1 amplification correlates with that of Mdm2 (6). On the other hand, AIB1 expression is also regulated by ubiquitination (59). However, other proteins that interact with p53 and/or ERα could also play important roles in regulating the accumulation of the receptor in breast cancer cells. A good candidate could be E6-AP, an E3 ubiquitin-ligase recruited by ERα (41, 29) which also targets p53 (50).

Our results dealing with the effect of stress-inducing agents on ERα expression clearly indicated that the increased turn-over of the receptor in the presence of the pure antiestrogen ICI182,780 involves different mechanisms than those implicated in the E2-dependent degradation. Similar dissociation between the effects of E2 and ICI182,780 were previously reported (2) and other pathways involving for example the NEDD8 ubiquitin-like protein (13) or CSN5/Jab1 (7) could account for the degradation in the presence of pure antihormones.

Previous data have highlighted the existence of a ligand-independent degradation of ERα and demonstrated the role of CHIP (*Carboxyl terminus of Hsc70-interacting protein*) in the ubiquitination of misfolded ERα (55). Our data suggest that, when overexpressed, Mdm2 is also involved in the ligand-independent turn-over of ERα. In these conditions, several evidences indicate that p53 could be dispensable for the effect of Mdm2 and this could reflect the fact that, upon overexpression of Mdm2, the binding equilibrium between ERα and Mdm2 could be strongly displaced towards complex formation thus avoiding the requirement for p53 to stabilize the interaction. Finally, although ubiquitination of ERα has previously been evidenced (58), the location of target residues is still unknown and further work will be require to define which particular lysine residues are modified by Mdm2 in the presence or absence of E2.

The present work also highlights the complex regulation of ERα gene expression by cellular stress. We first show that several stress-inducing agents which stabilize p53 also increase ERα levels and block its E2-dependent down-regulation. The stabilization of p53 upon treatment with stress-inducing agents results from its dissociation from Mdm2 due to post-translational modifications such as phosphorylation (36). It is interesting to note that phosphorylation of nuclear receptors has also been linked to their turn-over (25, 15 ,30). Concerning ERα, it has very recently been reported that S118 is an essential determinant of ERα degradation (57) and a previous work has shown that extracellular signal-regulated kinase 7 (ERK7) enhances the destruction of the receptor in a ligand-independent manner (17). By contrast, another report suggests that in MCF-7 cells, inhibition of MAPK results in an increased degradation of ERα (33). Altogether, these data suggest that phosphorylation is directly or indirectly involved in ERα turn-over. Interestingly we found that the degradation observed upon Mdm2 overexpression

was no longer detected when we tested ERα mutated on the phosphorylated serine 118 residues (data not shown).

Our data clearly indicated that all the stress-inducing agents that we used (MG132, UV, RITA) strongly inhibited ERα mRNA accumulation. Cellular stress produced therefore a dual effect on ERα protein accumulation, *i.e.* an early positive effect on ERα stability followed by a delayed negative regulation at the mRNA level (which led to a strong inhibition of receptor activity). Additional experiments will be needed to decipher the mechanisms which take place at the transcriptional or post-transcriptional levels and account for the negative regulation of ERα mRNA accumulation. One hypothesis could be that the increase in p53 levels (which occurs upon cellular stress) directly or indirectly participates in the repression of ERα mRNA accumulation. However, it has recently been suggested using stable transfection of antisense p53 or overexpression of p53 in MCF-7 cells that ERα transcription was positively regulated by p53 (3). Further work will be required to precise the mechanisms of stress-induced inhibition of ERα expression and to define its relevance in the efficacy of proteasome inhibitors in breast cancer therapy (44).

It has been suggested that the E2-dependent turn-over of ERα was required for efficient transactivation of the receptor (32). The data presented herein do not support such a conclusion. We first show that in p53/Mdm2-/- cells where E2 treatment led to an increase in ERα accumulation, we observed a strong hormone-dependent transactivation. In addition, in MCF-7 cells stably transfected with an ERE-containing reporter gene, the blockade of ERα E2-dependent degradation upon UV irradiation (or MG132, data not shown) did not abolish the transcriptional response to E2. Moreover, the same results were obtained on the endogenous E2-

regulated pS2 gene (data not shown) thus confirming the dissociation of stress-inducing agents on the accumulation of ERα and on its transactivation. Altogether, the present work dissociates the effect of E2 on ERα turn-over from the effect of hormone on transactivation, thus supporting previous studies performed using MG132 which suggested that the two events are not linked (14).

In conclusion, this study emphasizes the relevance of protein-protein interactions between nuclear receptors, the Mdm2 oncogene and the p53 tumor suppressor. Several physiological and pathological consequences of these interactions have been proposed for the glucocorticoid receptor (52). The involvement of an oncogenic E3-ligase in the regulation of ERα expression might appear surprising since this receptor is believed to mediate proliferative signaling of estrogens. However, our laboratory has shown that in the absence of ligand, ERα also exerts anti-invasive activity in breast cancer cell lines (46) and this effect could therefore be lowered in tumor cells overexpressing Mdm2. Mdm2 is frequently overexpressed in human cancers including breast carcinoma and ER-positive breast cancer cell lines express high levels of Mdm2 mRNA and protein compared to ER-negative cells. (Gudas CCR, 1995). It would be of great interest to quantify the level of Mdm2 protein in different breast tumors with changes in ERα expression. Altogether, the present work highlights the role of Mdm2 on hormone signaling and will be at the basis of future investigations to decipher its importance in various physio-pathological situations.

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FIGURE LEGENDS

Figure 1 - Interaction of ERα with p53 and Mdm2.

(A) GST pull-down assays were carried out as described in material and methods using bacterially expressed GST, GST-Mdm2 or GST-LBD proteins to retain ^{35}S-labelled ERα or Mdm2 in the presence of vehicle (C), 17β-estradiol (E2, 10^{-6}M) or 4-hydroxytamoxifen (OHT, 10^{-6}M). Inputs represent 10% of the material used in the assay. **(B)** Schematic representation of the ERα mutants. *In vitro* translated full-length ERα or various deletion mutants were analyzed for their interaction with the GST-Mdm2 fusion protein. **(C)** ERα and Mdm2 expression vectors were transfected into p53/Mdm2+/+ U2OS cells (lanes 1 and 2) or p53/Mdm2-/- mouse embryo fibroblasts (MEFdKO, lanes 3 and 4). Whole cell extracts were subjected to immunoprecipitation (lanes 2 and 4) as described in Materials and Methods using anti-Mdm2 antibody and Western-blotted using either anti-Mdm2 (upper panel) or anti-ERα antibody (lower panel). Whole-cell extracts are shown in lanes 1 and 3. **(D)** GST pull-down assays were carried out as in (A) using the bacterially expressed GST-C/D protein (containing the DBD of ERα) or GST alone to retain ^{35}S-labelled p53. **(E)** Mammalian two-hybrid assay was carried out using MCF-7 cells transfected with the 17M5-Luc reporter together with expressing vectors for Gal4 or Gal-Mdm2 with VP-16 or ER-VP16, in the presence or not of p53. Luciferase activity was expressed as % of control in presence of Gal4 and was the mean of three values.

Figure 2 - p53 and Mdm2 regulate E2-dependent expression of ERα.

(A) ERα accumulation was analyzed by Western-blot using an anti-ERα antibody. MEF WT and MEFdKO cells (p53/Mdm2-/-) were transfected with an ERα expression vector and treated 20h post-transfection with vehicle (C) or 17β-estradiol (E2, 10^{-8}M) for 20h. **(B)** Western-blot analysis of ERα accumulation in MEFdKO cells transiently transfected with an ERα expression vector (150ng) together with increasing amounts of p53 and Mdm2 expression plasmids (62.5, 125 and 250ng for each vector) or empty vectors alone. Cells were cultured 20h post-transfection in the presence or absence of 17β-estradiol (E2, 10^{-8}M). The asterisk indicated a non- specific band detected by anti-ERα antibody. **(C)** MEFdKO were transiently transfected either with the 17EB-Luc reporter plasmid (300ng) and ERα (100ng) alone or with increasing amounts of p53 and Mdm2 expression vectors (same transfection experiment as for the Western-Blot analysis shown in 2B). Cells were treated with vehicle (C) or 17β-estradiol (E2, 10^{-8}M) and the luciferase activity was quantified as described in Materials and Methods. Results were expressed relative to control in presence of ERα alone (% of control) and were the mean of three values. **(D)** HeLa cells were transfected either with ERα (1.5µg) or ERα and Mdm2 (1.5µg each) expression vectors and treated with 17β-estradiol (E2, 10^{-8}M) for 1h. Cycloheximide (CHX, 50µM) was added to inhibit ERα synthesis (time 0) and experiments were stopped at the indicated times. Mdm2 overexpression and ERα half-life were analyzed by Western-blot.

Figure 3 - Effect of Mdm2 on ligand-independent ERα expression.

(A) Either ERα or ERα and Mdm2 expression vectors were transfected (1.5µg each) into HeLa cells. ERα and Mdm2 levels were quantified by Western-Blot 24h after transfection. **(B)** HeLa cells were transfected either with ERα expression vector alone (1.5µg) or with ERα and increasing amounts of Mdm2 expression vector (150, 500 and 1500ng). ERα and Mdm2 levels were analyzed by Western-blot. **(C)** The stability of ERα protein was measured in the absence of ligand by pulse-chase assay with cycloheximide in HeLa cells transfected with ERα or with ERα and Mdm2 expression vectors (1.5µg each). The presence of exogenous Mdm2 protein was confirmed by Western-blot. **(D)** The 17EB-Luc reporter plasmid (500ng) was transiently transfected in HeLa cells together with ERα expression vector (150ng) in the presence or not of Mdm2 expression vector (1µg). The luciferase activity was quantified 20h after transfection. Results, expressed relative to control in presence of ERα alone (% of control), were the mean of three values.

Figure 4 - Mechanisms of Mdm2-dependent degradation of ERα.

(A) A GST pull-down experiment was performed to determine the *in vitro* interaction between ^{35}S-labelled Mdm2 or Mdm2ΔRING (deleted of the C-terminal region) with the GST-AF2 fusion protein which contains the ligand-binding domain of ERα. **(B)** HeLa cells were transfected either with the ERα expression plasmid alone (1.5µg) or together with Mdm2 or Mdm2ΔRING expression vector (1.5µg). The accumulation of ERα and Mdm2 proteins (wild-type or Mdm2ΔRING) was analyzed by Western-Blot. **(C)** HeLa cells were transiently transfected with ERα expression vector alone (500ng) (lanes 1, 2, 4 and 5) or together with the Mdm2 expression vector (1µg) (lane 3 and 6). Cells were treated with 17β-estradiol (E2, 10^{-8}M, lanes 2 and 5) or with vehicle alone (lanes 1, 3, 4 and 6). The expression of ERα and Mdm2 was analyzed by immunoblotting. The same experiment was performed using the wild-type receptor or the HE11 ERα mutant (deleted of the DBD).

Figure 5 - Effect of p53-inducing agents on ERα signaling.

(A) MCF-7 cells were treated for 20h with 10^{-7}M estrone (E1), 17β-estradiol (E2), estriol (E3) or diethylstilbestrol (DES) in the absence (control) or presence of MG132 (4μM). ERα and p53 protein levels were analyzed by Western-blot. **(B)** MCF-7 cells were cultured with MG132 (4μM), actinomycin D (10nM) or exposed to UV (150J/m^2) for 20h and apoptosis was measured by cytoplasmic nucleosomes quantification. Values were normalized by DNA quantification assay (measurement of DNA with DABA). **(C)** MCF-7 cells were irradiated (UV), treated with MG132 (4μM), actinomycin D (10nM) or untreated (control) in the presence of vehicle (C) or 17-estradiol (E2, 10^{-8}M). Extracts were prepared and Western-blotted with antibodies for ERα and p53. **(D)** MCF-7 cells were irradiated by UV or not (control) and cultured in the presence of vehicle (C), 17β-estradiol (E2, 10^{-8}M), hydroxytamoxifen (OHT, 10^{-8}M) or ICI182,780 (ICI, 10^{-8}M) for 20h. ERα and p53 accumulation were analyzed by Western-Blot. **(E)** Time course experiment with UV was performed in MCF-7 cells stably transfected with ERE-βglobin-Luciferase (MELN cells). Cells were treated with vehicle (control), 17β-estradiol (E2, 10^{-8}M), hydroxytamoxifen (OHT, 10^{-8}M) or ICI182,780 (ICI, 10^{-8}M) for 20h either 2, 4 or 8h post-irradiation or without being irradiated (0). Luciferase activity was measured as described in Materials and Methods and results expressed as relative activity (% of E2 without irradiation) were the mean (±SD) of three values.

Figure 6 - Effect of stress-inducing agents on ERα stability and activity.

(A) The steady-state level of ERα was measured by pulse-chase assay with cycloheximide (CHX). MCF-7 cells were irradiated with UV (lower panel) or not (upper panel), and treated concomitantly with 17β-estradiol for 4h. Cycloheximide was then added (time 0) during 2, 4 or 6h. ERα stability was measured by Western-Blot. **(B)** Quantification of the experiment shown in A. The intensity of the bands corresponding to ERα levels was determined by PCBAS imaging. Values were normalized by quantifying actin expression on the same blot. Results are expressed as % of control (time 0). **(C)** Kinetics of ERα and p53 accumulation by Western-Blot in stably transfected MELN cells treated or not with 17β-estradiol (E2, 10^{-8}M) in presence or not of RITA (1μM) during 2, 4 or 8h. **(D)** MELN cells were treated exactly as described in panel C and the luciferase activity corresponding to the stably integrated reporter gene was quantified. Results expressed as relative luciferase activity (% of control) were the mean (±SD) of three values.

Figure 7 – Stress-inducing agents decrease ERα expression at the mRNA level.

(A) MELN cells were treated with vehicle or 17β-estradiol (E2, 10^{-8}M) in the presence or not of RITA (1μM) for 12h. ERα mRNA levels were quantified by real-time quantitative RT-PCR as described in Materials and Methods. The results are expressed in arbitrary units after normalization using HPRT mRNA levels. Values are the means ± SD of three values. **(B)** Kinetics of ERα and p53 accumulation measured by Western-Blot in MELN cells treated with vehicle or 17β-estradiol (E2, 10^{-8}M) in the presence or not of RITA (1μM) for 24 or 48h. **(C)** MELN cells were treated exactly as in B, and the luciferase activity of the stably integrated reporter gene was quantified. Results were expressed as relative luciferase activity (% of control without stress) and were the mean of three values.