The antiestrogen fulvestrant (ICI 182,780) causes immobilization of estrogen receptor-α (ERα) in the nuclear matrix accompanied by rapid degradation by the ubiquitin-proteasome pathway. In this study we tested the hypothesis that fulvestrant induces specific nuclear matrix protein-ERα interactions that mediate receptor immobilization and turnover. A glutathione S-transferase (GST)-ERα-activating function-2 (AF2) fusion protein was used to isolate and purify receptor-interacting proteins in cell lysates prepared from human MCF-7 breast cancer cells. After SDS-PAGE and gel excision, mass spectrometry was used to identify two major ERα-interacting proteins, cytokeratins 8 and 18 (CK8-CK18). We determined, using ERα-activating function-2 mutants, that helix 12 (H12) of ERα, but not its F domain, is essential for fulvestrant-induced ERα-CK8 and CK18 interactions. To investigate the in vivo role of H12 in fulvestrant-induced ERα immobilization/degradation, transient transfection assays were performed using wild type ERα, ERα with a mutated H12, and ERα with a deleted F domain. Of those, only the ERα H12 mutant was resistant to fulvestrant-induced immobilization to the nuclear matrix and protein degradation. Fulvestrant treatment caused ERα degradation in CK8-CK18-positive human breast cancer cells, and CK8 and CK18 depletion by small interference RNAs partially blocked fulvestrant-induced receptor degradation. Furthermore, fulvestrant-induced ERα degradation was not observed in CK8 or CK18-negative cancer cells, suggesting that these two intermediate filament proteins are necessary for fulvestrant-induced receptor turnover. Using an ERα-green fluorescent protein construct in fluorescence microscopy revealed that fulvestrant-induced cytoplasmic localization of newly synthesized receptor is mediated by its interaction with CK8 and CK18. In summary, this study provides the first direct evidence linking ERα immobilization and degradation to the nuclear matrix. We suggest that fulvestrant induces ERα to interact with CK8 and CK18, drawing the receptor into close proximity to nuclear matrix-associated proteasomes that facilitate ERα turnover.

Estrogen receptor-α (ERα), a member of the nuclear receptor family, is a ligand-dependent transcription factor that mediates physiological responses to its cognate ligand, 17β-estradiol (E2), in estrogen target tissues such as the breast, uterus, and bone (1). Because ERα is a short-lived protein (half-life of 4–5 h), its cellular levels are strictly regulated (2). Although ERα turnover is a continuous process (2), dynamic fluctuations in receptor levels, mediated primarily by the ubiquitin-proteasome pathway (3–6), occur in response to changing cellular conditions (7–9). In addition, differing ligands have been demonstrated to exert differential effects on steady-state levels of ERα (10, 11). For example, E2 and the "pure" ERα antagonists (i.e. ICI 164,384, ICI 182,780, RU 58,668, and ZK-703) (12, 13) induce receptor turnover, whereas the "partial agonist/antagonist 4-hydroxytamoxifen (4-OHT) stabilizes ERα (14, 15). E2-mediated ERα degradation is dependent on transcription, coactivator recruitment, and new protein synthesis, whereas ICI-induced degradation of ERα is independent of these processes (16–18). Thus, although both E2 and pure antiestrogens induce ERα degradation, their mechanisms of action differ markedly. In addition to altering ERα stability and turnover, different ligands have been shown to have profoundly distinct effects on receptor mobility and cellular localization. For example, ERα was found localized exclusively in the nucleus after E2 and 4-OHT treatment, whereas ICI caused both nuclear and cytoplasmic receptor localization (13, 19). Stenoien et al. (20), using fluorescence recovery after photobleaching, demonstrated that E2, 4-OHT, and ICI treatment resulted in reduced nuclear mobility of ERα tagged with cyan fluorescent protein (20). In that study complete fluorescence recovery was not observed after ICI treatment due to immobilization of ERα to the nuclear matrix (20). Additional studies have further shown a rapid immobilization of the ERα-ICI complex within the nuclear matrix, with sequestration in a salt-insoluble, nuclear compartment (21, 22), although the precise nature of the receptor-nuclear matrix interaction remains unknown. Fulvestrant (faslodex, ICI 182,780) belongs to a new class of antihormonal therapy for advanced breast cancer called selective estrogen receptor down-regulators (SERDs) (23, 24). SERDs act as potent antagonists by inducing rapid receptor turnover and display no agonist activity in estrogen target tissues. SERDs differ markedly from the class of molecules called selective estrogen receptor modulators (SERMs), such as 4-OHT, that function as either agonists or antagonists, depending upon the target tissue (24). The pure antagonistic property of fulvestrant is due to a steroidal structure containing a long bulky side chain (25), which induces a distinct conformational change in the ligand binding domain of ERα (26), specifically in the position of helix 12 (H12), to prevent receptor dimerization and binding to DNA (27). Because specific mutations in H12 can reverse the pure antiestrogenic properties of fulvestrant (28, 29), H12 may contribute to fulvestrant-induced ERα degradation.

The abbreviations used are: ERα, estrogen receptor-α; CK, cytokeratin; E2, 17β-estradiol; GFP, green fluorescent protein; ICI, ICI 182,780; 4-OHT, 4-hydroxytamoxifen; siRNA, small interference RNA; SERD, selective estrogen receptor down-regulator; GAPDH, glyceraldehyde phosphate dehydrogenase; GST, glutathione S-transferase; AF2, activating function-2; wt, wild type; H12, helix 12.

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Antiestrogen-induced ERα Degradation

In this study we demonstrate that fulvestrant-induced ERα degradation by the ubiquitin-proteasome pathway was investigated. We show that this SERD induces specific ERα cytoskeleton CK8–CK18 interactions, the major intermediate filament proteins found in the nuclear matrix and cytoplasm of ERα-positive breast cancer cell lines (30). We further demonstrate that H12 is essential for these cytoskeleton interactions and, subsequently, receptor immobilization within the nuclear matrix. Furthermore, we show that fulvestrant-mediated receptor degradation and cytoplasmic localization correlate directly with CK8 and CK18 levels in breast cancer cells. Because proteinases have been shown to be associated primarily with intermediate filaments (31–33), we suggest that fulvestrant induces specific receptor-cytokeratin interactions in the nuclear matrix, bringing ERα into close proximity to proteinases for subsequent degradation.

EXPERIMENTAL PROCEDURES

Materials—The following antibodies and reagents were used in this study: anti-ERα (HC20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or monoclonal anti-human ERα (Chemicon International, Inc., Temecula, CA); monoclonal anti-human cytokeratin 8 (RCK102; BD Biosciences) and monoclonal anti-human cytokeratin 18 (RCK106; BD Biosciences); monoclonal anti-cytokeratin peptide 8 (Sigma); mouse anti-glyceraldehyde phosphate dehydrogenase (GAPDH) (Chemicon International); glutathione-Sepharose 4 Fast Flow beads (Amersham Biosciences); SuperSignal West Pico chemiluminescent substrate (Pierce); protease inhibitor mixture set III (Calbiochem-Novabiochem); Lipo-nectam Plus reagent, Geneticin, and cell culture reagents (Invitrogen); FuGENE (Roche Applied Science); 4-OHT and MG132 (Sigma); ICI 182,780 (Tocris Cookson Ltd., Ellisville, MO); RNase-free DNase I and Bl2L (DE3)pLysS competent cells (Promega, Madison, WI).

Plasmid Construction—Wild type ERα pSG5-ERα(HEGO) was kindly provided by Dr. Pierre Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France) and GFP-ERα (26) by Dr. Michael Mancini (Baylor College of Medicine, Houston, TX). The ERα helix 12 mutant pRST-7-hER3X (D538N/E542Q/D545N) was kindly provided by Donald McDonnell (Duke University, Durham, NC). pGEX-6P-1-AF2, pGEX-6P-1-AF2ΔAF, pGEX-6P-1-AF2ΔAFH12, and pGEX-6P-1-ERα3X-AF2 were constructed by inserting the PCR fragment of interest into pGEX-6P-1 (BachH and Xhol site). pcDNA3-ERα3F, pcDNA3-ERα3XΔF, and pcDNA3-ERα3FΔH12 were generated by inserting the specific PCR DNA fragment into pcDNA3MyHisA (BachH and Xhol site). pcDNA3-CK8 was generated by inserting the CK8 PCR DNA fragment into pcDNA3MyHisA (BachH and Xhol site). pcDNA3-CK8 was generated by inserting CK8 PCR DNA fragment into pcDNA3MyHisA (EcoRI and Xhol site). Cloning results were confirmed by subjecting all constructs to DNA sequencing.

Cell Lines—The human cervical carcinoma HeLa cell line and the breast cancer cell lines MCF-7 and its daughter, C4-12 (ERα-negative, CK8- and 18-positive (34)), are routinely maintained in our laboratory, as described previously (9, 35). MDA-MB-231 and T47D breast cancer cells were purchased from ATCC (Manassas, VA). MDA-MB-231 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. T47D cells were maintained in RPMI 1640 medium 2 mM L-glutamine, 1.0 mM sodium pyruvate, 50 units/ml penicillin, 50 μg/ml streptomycin, 10 mM Hepes, 6 ng/ml insulin, and 10% fetal bovine serum. Before experiments involving transient transfection and hormone treatment, cells were cultured in hormone-free medium (phenol red-free minimum Eagle’s medium (MEM) with 5% charcoal-stripped fetal bovine serum) for 3 days.

Stable Transfection of ERα—C4-12 or HeLa cells were transfected with pcDNA-ERα (C4-12/ERα and HeLa/ERα, respectively) using Lipofectamine Plus Reagent and exposed to antibiotic (G418; 0.5 mg/ml) for 3 weeks. Expression of ERα in G418-resistant colonies was verified by immunoblotting with anti-ERα.

Transient Transfection Assay—T47D and HeLa cells were cultured in hormone-free medium for 3 days and transfected with equal amounts of total plasmid DNA (adjusted by the corresponding empty vectors) using Lipofectamine Plus reagent or FuGENE according to the manufacturer’s guidelines. Five hours later, the DNA/Lipofectamine mixture was removed, and cells were cultured in hormone-free medium. Unless stated otherwise, 24 h after transfection, cells were treated with the specified drug.

RNA Interference (siRNA)—siRNA transfection reagent, control siRNA, CK8 siRNA, and CK18 siRNA were purchased from Santa Cruz Biotechnology. The CK8 and CK18 siRNAs (singly or both) were transfected into MCF-7 cells according to the manufacturer’s protocol; 72 h after transfections, cells were treated with 100 nM ICI 182,780. Whole cell lysates were prepared in 1X SDS sample buffer. Protein levels were examined by Western blotting using specific antibodies.

Preparation of Whole Cell Extracts—Whole cell extracts were prepared by suspending cells in SDS lysis buffer (62 mM Tris, pH 6.8, 2% SDS, 10% glycerol, and protease inhibitor mixture III). After 15 min of incubation on ice, extracts were sonicated, insoluble materials were removed by centrifugation (15 min at 12,000g), and supernatant protein concentrations were determined using a Bio-Rad protein assay kit.

Preparation of Nuclear Extracts and Nuclear Matrix—Nuclear extract was prepared using a nuclear extraction kit (Active Motif, Carlsbad, CA), according to the manufacturer’s protocol. Nuclear matrix was prepared following the procedure described by Coutts et al. (30). Briefly, cell nuclei were prepared with nuclear matrix buffer (100 mM NaCl, 300 mM sucrose, 10 mM Tris–HCl, pH 7.4, 2 mM MgCl2, 1% (v/v) thioglycol containing 1 mM phenylmethylsulfonyl fluoride and 0.5% (v/v) Triton X-100). Nuclei were resuspended in digestion buffer (50 mM NaCl, 300 mM sucrose, 10 mM Tris–HCl, pH 7.4, 3 mM MgCl2, 1% (v/v) thioglycol, 0.5% (v/v) Triton X-100), digested with DNase I (168 units/ml) for 20 min at room temperature, and then sequentially extracted using 0.25 M ammonium sulfate and 2 mM NaCl. Nuclear matrix was resuspended in 1X SDS sample buffer and sonicated.

Western Blot and Quantitation—Whole cell lysates were prepared in 1X SDS sample buffer by sonication, and total protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. ERα levels were determined by Western blot using a LI-COR (Lincoln, NE) imaging system. The membrane was incubated with primary antibody followed by incubation with infrared dye IR800-labeled goat anti-mouse IgG or IR700-labeled goat anti-rabbit IgG (LI-COR) secondary antibodies and quantitated with LI-COR Odyssey software. For immunoblotting by enhanced chemiluminescence (ECL), primary antibody was detected by horseradish peroxidase-conjugated second antibody and visualized using an enhanced SuperSignal West Pico chemiluminescent substrate.

GST Pull-down Assay—GST pull-down assays were performed as we have described previously (35, 36). To fuse ERα-AF2 with GST, an ERα-AF2 PCR fragment (amino acids 297–595) was cloned into the BamH I and Xhol sites of the plasmid pGEX-6P-1 and subjected to DNA sequencing to confirm the correct reading frame. The GST-tagged AF2 was then expressed in Bl2L cells and purified as described (36, 37).
Briefly, overnight cultures of BL21 cells containing the plasmid pGEX-6P-1–GST-ERα-AF2 were diluted (1:20), cultured in fresh medium for 2 h, and treated with 0.1 mM isopropyl β-D-thiogalactoside for 3 h. Induced bacteria were then collected by centrifugation and lysed in NETN buffer containing 0.5% Nonidet P-40, 1 mM EDTA, 20 mM Tris, pH 8.0, 100 mM NaCl, and protease inhibitors. GST-ERα-AF2 was purified on glutathione-Sepharose 4 Fast Flow beads (Amersham Biosciences). MCF-7 cell lysates were prepared by sonicating cells in cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, pH 7.5). Whole cell lysates were then incubated with the glutathione-bound GST-ERα-AF2 in binding buffer (60 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 7.5, 0.05% Nonidet P-40, 1 mM dithiothreitol, 6 mM MgCl₂, and 8% glycerol) in the absence or presence of corresponding ligands or vehicle for 3 h at 4°C. After washing with binding buffer, ERα-AF2-bound proteins were eluted, separated by 10% SDS-polyacrylamide, and visualized by Coomassie Blue. Specific proteins were cut from the gel, eluted, and analyzed by MALDI and liquid chromatography mass spectrometry by the Indiana University Protein Analysis Research Center (Indianapolis, IN).

**RESULTS**

**Fulvestrant Induces ERα-Intermediate Filament Protein Interactions**—Previously it was shown that treatment of breast cancer cells with the pure antagonist ICI resulted in ERα immobilization and resistance to biochemical extraction within the nuclear matrix (21). For this study we hypothesized that fulvestrant-dependent ERα-interacting proteins in the nuclear matrix were responsible for this phenomena. To identify putative fulvestrant-dependent ERα-interacting proteins, cell lysates from human breast cancer MCF-7 cells were incubated with immobilized GST-ERα-activating function-2 (AF2) in the presence of ICI. Interacting proteins were eluted from the beads, separated by SDS-PAGE, and stained with Coomassie Blue. Fulvestrant-specific interacting protein bands (Fig. 1A) were excised from the gel and subjected to mass spectrometry (MALDI and liquid chromatography mass spectrometry) analysis, resulting in two of the proteins being identified as cytokeratins 8 and 18 (CK8 and CK18). To validate those findings, Western blot analysis using CK8- or CK18-specific antibodies, was performed to permit conclusive identification of these putative ERα binding partners (Fig. 1, B and C). No interaction between ERα and CK8 or CK18 was observed in the presence of either E2 or 4-OHT (Fig. 1). These ERα-CK8-CK18 associations were also stable in the presence of high salt (Fig. 1, last lane), consistent with other reports that ERα is insoluble after immobilization by ICI or RU 58628 (21, 38). To further demonstrate the interaction in vivo, co-immunoprecipitation was performed using MCF-7 whole cell lysates and an ERα-specific antibody in the absence or presence of fulvestrant. As shown in Fig. 1D, CK8 and CK18 were seen in the ERα complex only in the presence of ICI, suggesting that fulvestrant induces an endogenous interaction between ERα and CK8-CK18.

**Expression of CK8-CK18 in ERα-positive and -negative Cancer Cell Lines**—It has been previously shown that both CK8 and CK18 are nuclear matrix-intermediate filament proteins present in ERα-positive cells (30). To investigate whether a correlation exists between expression of ERα and CK8-CK18, whole cell lysates were prepared from human breast (MCF-7, T47D, MDA-MB-231) and cervical cancer (HeLa) cell lines. Levels of CK8 and CK18 protein were analyzed by Western blot analysis. Differential CK expression was observed between the ERα-positive and -negative cell lines (Fig. 2A). Furthermore, CK8 and CK18 protein levels were markedly higher in MCF-7 and T47D (ERα-positive) cells as compared with the ERα-negative MDA-MB-231 and HeLa cells.

**Effect of Fulvestrant on the Association of ERα with the Nuclear Matrix and Receptor Degradation**—Distinct ligands can specifically affect ERα extractability from the nucleus of breast cancer cells (38). To further characterize the association between ERα and the nuclear matrix in the presence of antiestrogens, MCF-7 and T47D cells (ERα-, CK8-, and CK18-positive) were treated with ICI or 4-OHT followed by isolation of nuclear matrix fractions. Nuclear matrix prepared from MDA-MB-231 (ERα-negative; CK8- and CK18-positive, Fig. 2A) was used as a control.
In the nuclear matrix of ERα-positive cells, CK8 and CK18 were highly abundant (Fig. 2C, upper panel, Coomassie Blue; middle panel, Western blot). In the presence of ICI, the majority of ERα protein was unextractable and remained tightly associated with the nuclear matrix (Fig. 2C); in contrast, in the presence of 4-OHT, ERα was loosely associated with the nuclear matrix, readily extractable, and thus, more abundant in the nuclear extract (Fig. 2C, bottom panel). These observations are consistent with the result that fulvestrant induces a salt-resistant ERα-C8 and CK18 interaction (Fig. 1) and that ERα extractability varies in the presence of different ligands (38).

To monitor ERα immobilization and degradation, nuclear extract and nuclear matrix were prepared from MCF-7 cells treated with fulvestrant for 0–4 h. As shown in Fig. 2B, rapid (<30 min) immobilization of ERα from the nuclear extract to the nuclear matrix was observed followed by receptor degradation 1 h after the onset of ICI treatment. In addition, CK8 and CK18 were both localized in the insoluble nuclear matrix (Fig. 2B). Taken together, these observations demonstrate that after treatment with fulvestrant, ERα is rapidly sequestered in a salt-insoluble nuclear compartment before being degraded.

**Helix 12 Is Required for Fulvestrant-dependent Interaction of ERα with CK8 and CK18 and Antiestrogen-induced Immobilization of ERα to the Nuclear Matrix and Receptor Degradation**—Previous studies have suggested a role of two domains of ERα in ICI-induced receptor immobilization and degradation; that is, H12 and the F domain. Furthermore, Katzenellenbogen and coworkers (29) showed that mutations in H12 conferred resistance to ICI-induced degradation. Furthermore, to examine whether these two domains are required for fulvestrant-dependent interactions with CKs, several ERα AF2 mutant GST fusion proteins were constructed; AF2ΔF, with the F domain of AF2 deleted, AF2ΔFΔH12, completely lacking both F domain and helix 12, AF2–3X, with 3 mutated amino acids in H12 (D538N/E542Q/D545N), AF23XΔF, containing H12 mutations and lacking the F domain (Fig. 3A). In the presence of fulvestrant, the F domain deletion constructs remained capable of interacting with both CK8 and CK18, demonstrating that the F domain is not required for the ERα-CK interaction (Fig. 3B). However, removal of H12 or point mutations introduced into this region completely abolished fulvestrant-induced receptor-CK8-CK18 interactions (Fig. 3B). Interestingly, no interaction between ERβ and either CK8 or CK18 was observed after ICI treatment (Fig. 3B, last lane, ERβAF2). In MCF-7 cells (39, 40) and rat efferent ductules (40), ERβ appears to be resistant to fulvestrant-induced degradation, and our results further indicate that the lack of CK interactions may play a role in the inability of fulvestrant to degrade this ER isoform.

Having demonstrated that H12 is required for fulvestrant-induced interaction of ERα with CK8 and CK18, it was of interest to test whether H12 and the F domain are required for ERα immobilization. Plasmids containing wild type ERα (wtERα), ERαAFΔF, or ERα3X were transfected into the MDA-MB-231 breast cancer cell line (ERα-negative; CK8- and CK18-positive, Fig. 2A). Transfected MDA-MB-231 cells were treated with ICI or E2 for 30 min (this short treatment duration causes ERα immobilization but not degradation). Whole cell lysates and nuclear extracts were prepared, and ERα protein levels were determined by Western blot analysis. After E2 treatment, both wtERα and ERαAFΔF were extractable by nuclear extraction buffer (Fig. 3C), however, after treatment with ICI, neither construct was extractable (Fig. 3C). No effect of E2 or ICI on the extractability of the mutant ERα3X was observed (Fig. 3C). Taken together, these results indicate that H12 is essential for fulvestrant-induced immobilization of ERα to the nuclear matrix.

It was recently demonstrated that mutations in H12 could influence tamoxifen-mediated ERα stability (41). To examine whether H12 contributes to fulvestrant-mediated receptor degradation, T47D breast cancer cells (CK8- and CK18-positive, Fig. 2A) were transiently transfected with full-length ERα3X (point-mutated helix 12) or wtERα. Receptor levels were assessed by Western blot analysis after treatment with ICI for 1 h. As shown in Fig. 3D, degradation of wtERα, but not ERα3X, was observed after ICI treatment, suggesting that an intact H12 is required for fulvestrant-induced ERα degradation.
Because the F domain of ERα contains a PEST sequence (residues 555–567), a proposed signal for rapid intracellular breakdown of proteins (42), it was of interest to investigate whether this domain may be involved in fulvestrant-induced ERα degradation. T47D cells were transiently transfected with plasmids expressing ERαF, ERα3X, or ERαΔFΔH12 and treated with ICI for 1 h. The relative stability of each mutant ERα was then assessed using Western blot analysis using a monoclonal antibody against the N-terminal region of ERα, which recognizes receptors with C-terminal deletions. As shown in Fig. 4, a decrease in the level of ERαΔF protein was observed after ICI treatment;

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in contrast, both ERα3XΔF and ERαΔFΔH12 were resistant to fulvestrant-induced degradation. Moreover, ERα3XΔF levels actually increased after treatment with the antiestrogen, likely due to blockage of basal turnover of the mutant receptor (Fig. 4). In support of this possibility, treatment with the proteasome inhibitor MG132, an inhibitor of basal ERα protein turnover (43) increased levels of ERα protein (Fig. 4, A and B). Collectively, these results indicate that the F domain is not required for fulvestrant-induced ERα degradation, in contrast to H12. Our observations also support those of Pakdel et al. (43), who reported that the F domain is dispensable for E2-induced degradation of ERα (43).

Fulvestrant-induced Degradation of ERα Is Dependent on Cellular Levels of CK8 and CK18—Having established that fulvestrant induces an interaction between ERα, the nuclear matrix, and CK8 and CK18, it was important to define the role of these intermediate filaments in antiestrogen-mediated receptor degradation. To test receptor stability in the presence or absence of these CKs, we utilized C4-12 cells, an ERα-negative, CK8-CK18-positive breast cancer cell line derived from MCF-7 (34) and HeLa cells (negative for ERα, CK8, and CK18). These cell lines were stably transfected with wtERα and treated with ICI for 1–4 h; ERα protein levels were then measured by Western blot. After treatment with ICI, marked degradation of ERα was observed in C4-12 cells (Fig. 5A) but not in HeLa cells (Fig. 5B), indicating that the presence of CK8 and CK18 is essential for receptor turnover by the pure antiestrogen. To investigate the effect of CK8-CK18 overexpression on fulvestrant-induced ERα degradation, HeLa cells (negative for CK8-CK18 and ERα) were co-transfected with CK8 and CK18 ( singly or both) along with ERα, and the transfected cells were treated with ICI for 2 h. ERα protein levels were subsequently determined by immunoblot analysis. As shown in Fig. 6A, overexpression of CK8-CK18 restored the ability of fulvestrant to degrade ERα in HeLa cells. We then examined whether fulvestrant-induced ERα degradation could be inhibited by CK8-CK18-specific small interference RNAs (siRNA). MCF-7 cells (CK8-CK18-positive) were transfected with CK8 or CK18 siRNAs ( singly or both) and treated with ICI for 2 h. CK8-CK18 and ERα protein levels were measured by Western blotting. As shown in Fig. 6B, CK8-CK18 siRNAs decreased the level of CK8 and CK18, and fulvestrant-induced ERα degradation was less dramatic in these MCF-7 cells.

Cytoplasmic Localization of ERα Is Associated with CK8 and CK18—A unique but poorly understood property of pure antagonists like the ICI compounds (13, 19) and RU 58668 (44) is the induction of cytoplasmic localization of ERα. Intermediate filament proteins CK8 and CK18 have been shown to be located in both the nuclear matrix as well as in the cytoplasm (30). To investigate whether fulvestrant-mediated cytoplasmic localization of ERα is associated with CK8 and CK18, we transfected an ERα-GFP plasmid into CK8- and CK18-positive or -negative cell lines (MCF-7, T47D, or HeLa cells, respectively; Fig. 2). Transfected cells were then treated with ICI in the presence or absence of the protein synthesis inhibitor cycloheximide or the partial antagonist 4-OHT. In untreated cells and cells treated with 4-OHT, expression of ERα-GFP was exclusively nuclear (Fig. 7, first and last columns, respectively). After treatment of MCF-7 and T47D cells with ICI, dramatic cytoplasmic localization of ERα was observed (Fig. 7, second column). This was completely blocked by cycloheximide treatment (Fig. 7, third column), consistent with a previous report demonstrating the requirement of new protein synthesis for fulvestrant-induced cytoplasmic ERα localization (44). In contrast to observations in MCF7 and T47D cells, in HeLa cells treated with fulvestrant markedly less cyto-
plasmic localization was observed based on both the percentage of cells displaying ERα-GFP in the cytoplasm and cytoplasmic ERα-GFP intensity (Fig. 7, last row). After 8 h of ICI treatment, most (>50%) MCF-7 and T47D cells showed some degree of cytoplasmic localization; however, <10% of the CK8-CK18-negative HeLa cells displayed cytoplasmic localization, in agreement with a previous report (19). Collectively, these results indicate that the presence of CK8 and CK18 is necessary for fulvestrant-induced cytoplasmic localization of ERα.

DISCUSSION

The antiproliferative effects of fulvestrant (ICI 182,780) on breast cancer cells are due to rapid degradation of ERα protein (12, 21). While the drug acts by immobilizing ERα to the nuclear matrix followed by rapid receptor turnover, the molecular mechanism has not been fully established. In this study we identified two fulvestrant-dependent ERα-interacting proteins, CK8 and CK18, members of the nuclear matrix intermediate filament family of structural proteins (30). We show that CK8 and CK18 are involved in fulvestrant-induced ERα immobilization and degradation, and we further demonstrate that H12 of ERα is essential for the fulvestrant-dependent interaction with CK8 and CK18. Although ERα has long been known to associate with the nuclear matrix (45), our findings are the first demonstration of fulvestrant-dependent interaction between ERα and intermediate filament proteins in the nuclear matrix. Because proteasomes are closely associated with intermediate filaments (31–33), we suggest that SERD-induced rapid degradation of ERα is due to specific interactions with CK8 and CK18 by
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bringing the receptor into close proximity to the 26 S proteasome protein degradation machinery.

Pure antiestrogens, like fulvestrant, can be converted to full estrogen agonists by specific mutations in H12 (28,29). H12 makes up most of the C-terminal helix within the ligand binding domain of ERα (46) and appears to be required for recruiting coactivators and corepressors, serving as a “molecular switch” that connects ligands with coregulators (47). This helix is required for ICI-induced immobilization, as demonstrated by Stenoien et al. (20) using fluorescence recovery after photobleaching, and mutations in H12 can abrogate E2-mediated degradation (3–6), suggesting that the H12 coactivator binding surface is required for ligand-mediated ERα down-regulation. Furthermore, antiestrogens have been shown to change ERα stability by altering the position of H12 (26). To test whether H12 is essential for receptor-CCK8 and -CCK18 interactions and, thus, the ability of fulvestrant to immobilize and degrade ERα, we examined the interaction between several GST-ERα-AF2 mutants and CCK8 and CCK18. Point mutations or deletion of H12, but not loss of F domain function, abolished CCK8 and CCK18 interactions, demonstrating that the F domain is not required for fulvestrant-induced ERα immobilization. Based on these results, we suggest that in the presence of fulvestrant, H12 interacts with CCK8 and CCK18 and immobilizes ERα within the nuclear matrix for subsequent degradation.

Because the interaction of ERα with CCK8 and CCK18 is specific for fulvestrant, it is likely that H12 assumes a different position when bound by ICI, as compared with 4-OHT, resulting in receptor degradation versus stabilization. Indeed, a recent report showing differences in antiestrogen-induced relocation of hydrophobic residues in H12 strongly supports this possibility (26). Of the ERα antagonists examined, ICI caused the greatest exposure of surface hydrophobicity, whereas 4-OHT caused the least exposure (26). Thus, it seems plausible that ICI induces a conformational change that allows H12 to interact with CCK8 and/or CCK18. Nonetheless, it is not clear how an ERα-CCK8-CCK18 interaction triggers rapid receptor turnover; however, proteasomes have recently been shown to be closely associated with intermediate filament proteins and, thus, likely facilitate this process (31–33).

It has previously been shown that pure antiestrogens (ICI 182,780, RU 58668) can disrupt ERα nucleocyttoplasmic shuttling and cause receptor cytoplasmic localization (13), a process that requires new protein synthesis (19). It is also known that both CCK8 and CCK18 are located in the cytoplasm and the nuclear matrix (30). In the present study, ERα cytoplasmic localization was observed only in CCK8-CCK18-positive cells, suggesting that these intermediate filaments play a role in retaining ERα in the cytoplasm after fulvestrant treatment. In support of this hypothesis, Htun et al. (19) reported that cytoplasmic retention of ERα varied between breast cancer cell lines, with greater cytoplasmic localization seen in ERα-positive MCF-7 and T47D cells as compared with ERα-negative MDA-MB-231 cells. Although an explanation for this observation was not offered (19), our findings that CCK8 and CCK18 are differentially expressed in these cell lines provides a plausible rationale. Interestingly, whereas other cytoskeletal proteins are present in the nuclear matrix (e.g. CCK-CCK9), these do not interact with ERα, as the basis for the specificity of ERα for CCK8 and CCK18 remains unclear.

Although it is well established that the level of ERα in breast tumors is a valuable predictor of a patient’s response to antiestrogen therapies such as tamoxifen and fulvestrant (48), CCK8 and CCK18, via their correlation with tumor differentiation (49), have also been used in cancer diagnosis. Furthermore, up-regulation of CCK8-CCK18 expression was associated with good prognosis in breast cancer patients (49, 50), whereas their down-regulation was correlated with a poor clinical outcome (51). We have previously shown that breast cancer cells with a disrupted ubiquitin-like NEDD8 pathway can acquire antiestrogen resistance (8) and that tumors from patients who developed resistance to fulvestrant can retain ERα expression (52). Taken together, it seems reasonable to suggest that disruption of ERα degradation may contribute to fulvestrant-resistant breast cancer. Because CCK8 and CCK18 are associated with fulvestrant-mediated ERα degradation, their decreased levels would likely disrupt fulvestrant-mediated ERα immobilization and degradation, which are both essential for the antiproliferative activity of this antiestrogen (8). Thus, we speculate that down-regulation of CCK8-CCK18 may be involved in fulvestrant resistance; furthermore, a H12 mutant ERα would likely be resistant to fulvestrant-mediated degradation, supporting the observation that H12 mutations can contribute to endocrine-resistant breast cancer (53–56). In conclusion, fulvestrant resistance is clearly multifactorial. We are currently investigating the role of the NEDD8 pathway and the nuclear matrix proteins CCK8 and CCK18 in antiestrogen-resistant breast cancer.

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Fulvestrant (ICI 182,780)-dependent Interacting Proteins Mediate Immobilization and Degradation of Estrogen Receptor-α
Xinghua Long and Kenneth P. Nephew


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