Ligand-dependent switching of ubiquitin–proteasome pathways for estrogen receptor

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Recent evidence indicates that the transactivation of estrogen receptor α (ER α) requires estrogen-dependent receptor ubiquitination and degradation. Here we show that estrogen-unbound (unliganded) ER α is also ubiquitinated and degraded through a ubiquitin–proteasome pathway. To investigate this ubiquitin–proteasome pathway, we purified the ubiquitin ligase complex for unliganded ER α and identified a protein complex containing the carboxyl terminus of Hsc70-interacting protein (CHIP). CHIP preferentially bound to misfolded ER α and ubiquitinated it to induce degradation. Ligand binding to the receptor induced the dissociation of CHIP from ER α . In CHIP $^-$ cells, the degradation of unliganded ER α was abrogated; however, estrogen-induced degradation was observed to the same extent as in CHIP $^{+/+}$ cells. Our findings suggest that ER α is regulated by two independent ubiquitin–proteasome pathways, which are switched by ligand binding to ER α . One pathway is necessary for the transactivation of the receptor and the other is involved in the quality control of the receptor.

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Introduction

The effects of estrogen are mediated through the estrogen receptors ER α and ER β , which function as ligand-induced transcriptional factors and belong to the nuclear receptor superfamily (Beato *et al*, 1995; Mangelsdorf *et al*, 1995; Chambon, 1996; McKenna and O'Malley, 2002). Estrogen binding to its receptor induces the ligand-binding domain

(LBD) to undergo a characteristic conformational change, whereupon the receptor dimerizes, binds to DNA and subsequently stimulates the gene expression. ER α is stimulated by two distinct activation regions, activation function-1 (AF-1) and AF-2, which are located in the C-terminal LBD and exert ligand-dependent transcriptional activity. Cellular response to estrogen is tightly controlled, and a large number of ER α -interacting proteins have been described as coactivators or corepressors that modify ER α transcriptional activity (Shang *et al*, 2000; Yanagisawa *et al*, 2002; Metivier *et al*, 2003).

Crystal-structural analysis of ER α and other nuclear receptors has revealed the presence of 12 conserved helices in their LBD (Shiau *et al*, 1998). The LBD forms a structure described as a sandwich of 12 α -helices (Helices 1–12) with a central hydrophobic ligand-binding pocket. Helix 12, the most C-terminal of these helices, has been identified as the critical core (AD core) of the AF-2 function of the receptor and plays an important role in coactivator binding to the ligand-bound receptor. In the presence of the ligand, the hinge region between Helices 11 and 12 moves closer to Helices 3 and 5, and Helix 12 is positioned over the ligand-binding pocket formed by Helices 3–5. The repositioned Helix 12 forms a hydrophobic groove with Helices 3 and 5. This hydrophobic groove is known to be important for the interaction with LXXLL motifs found in coactivator molecules (Heery *et al*, 1997).

The activation of nuclear receptors appears to be coupled with the degradation of these proteins by the ubiquitin–proteasome pathway (Boudjelal *et al*, 2000; Dace *et al*, 2000; Blanquart *et al*, 2002). Several recent studies have focused on the involvement of the ubiquitin–proteasome pathway in the estrogen-dependent degradation of ER α , which can be blocked with specific inhibitors of proteasome function, such as MG132 and lactacystin. It has also been reported that the 26S proteasome is essential for estrogen-dependent ER α transcription activity (Nawaz *et al*, 1999a; Lonard *et al*, 2000; Reid *et al*, 2003). Furthermore, several components of the ubiquitin–proteasome pathway have been identified as nuclear receptor-interacting proteins, including SUG1/TRIP1 (Lee *et al*, 1995), RSP5/RPF1 (Imhof and McDonnell, 1996), E6-AP (Nawaz *et al*, 1999b) and UBC9 (Poukka *et al*, 1999). These observations suggest that the ubiquitin–proteasome pathway may play an important role in regulating nuclear receptor levels and restricting the duration and magnitude of receptor activity in response to ligands. Nonetheless, mechanisms governing ER α protein levels remain poorly understood.

Here we show that, in the absence of estrogen, ER α is also ubiquitinated and degraded via a ubiquitin–proteasome pathway. The observation that estrogen-dependent ubiquitination of the receptor required the AD core region within the ER α LBD, whereas the ubiquitination of the unliganded receptor did not, raised the possibility that the ubiquitin ligase for unliganded ER α might differ from the ligase involved in estrogen-dependent ubiquitination. Therefore, we purified

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the ubiquitin-ligase complex for unliganded ER α and identified a chaperone complex containing the carboxyl terminus of Hsc70-interacting protein (CHIP) (Ballinger *et al*, 1999; Dai *et al*, 2003). CHIP selectively bound to and ubiquitinated misfolded ER α and stimulated the degradation of these receptors. This model was further supported by an experiment using CHIP-deficient mouse (CHIP $^{-/-}$) embryonic fibroblast cells. The unliganded ER α was degraded in CHIP $+/+$ cells but not in CHIP $-/-$ cells under thermally stressed conditions. In contrast, estrogen-dependent degradation was observed in both CHIP $+/+$ and CHIP $-/-$ cells, supporting the idea that the inactive and active forms of the receptor are regulated by two independent ubiquitin-proteasome pathways. Our findings shed light on the ubiquitin-proteasome network regulating nuclear receptors.

Results

Unliganded ER α is degraded through a ubiquitin-proteasome pathway

As shown in Figure 1A, addition of estrogen to MCF-7 cells reduced the level of ER α protein. The reduction of ER α was inhibited by the proteasome inhibitors MG132 or lactacystin. In the absence of estrogen, MG132 or lactacystin treatment also resulted in ER α accumulation (Figure 1A, lanes 3 and 5), suggesting that not only estrogen-bound ER α but also unliganded ER α is degraded through proteasomes. In ubiquitination assay, ER α was ubiquitinated in both the presence and absence of estrogen (Figure 1B, lanes 3 and 4), indicating that this process is mediated through ubiquitin-proteasome pathways.

We next determined whether the degradation of unliganded and liganded ER α is regulated by the same ubiquitin-proteasome pathway. It has been reported that truncated ER α , ER $\alpha\Delta$ AD, which does not have an AD core domain, does not exhibit estrogen-dependent degradation (Lonard *et al*, 2000). Thus, we examined the ubiquitination and degradation of ER $\alpha\Delta$ AD. ER α and ER $\alpha\Delta$ AD were transfected into 293 cells and the ER α protein level was examined by Western blot analysis. While the ER α degradation was observed regardless of estrogen treatment, ER $\alpha\Delta$ AD was stabilized by ligand binding, as it accumulates in response to estrogen. MG132

treatment increases the levels of ER $\alpha\Delta$ AD in the absence of the ligand but does not affect its estrogen-induced accumulation (Figure 1C). We next tested whether ER $\alpha\Delta$ AD turnover is

Figure 1 Unliganded ER α was degraded through a ubiquitin-proteasome pathway. (A) ER α was degraded in the absence of estrogen. The MCF-7 cells were cultured in the presence or absence of estrogen (10^{-8} M), or the proteasome inhibitor MG132 or lactacystin (10^{-6} M). ER α level was analyzed by Western blotting using anti-ER α monoclonal antibody. (B) ER α was ubiquitinated in the absence of estrogen. MCF-7 cells were cultured in the presence or absence of estrogen (10^{-8} M) or MG132 (10^{-6} M). ER α was immunoprecipitated using anti-ER α antibody. The ubiquitination status of ER α was analyzed by Western blotting using anti-ubiquitin antibody. (C) ER $\alpha\Delta$ AD was selectively degraded in the absence of estrogen. 293 cells were transfected with either ER α or ER $\alpha\Delta$ AD (500 ng). At 24 h post-transfection, the cells were cultured in the presence or absence of estrogen (10^{-8} M) or MG132 (10^{-6} M). ER α or ER $\alpha\Delta$ AD protein levels were analyzed by Western blotting using anti-ER α antibody. (D) ER $\alpha\Delta$ AD was ubiquitinated in the absence of estrogen. Flag-tagged ER $\alpha\Delta$ AD (500 ng) was transfected into 293 cells in the presence or absence of estrogen (10^{-8} M) or MG132 (10^{-6} M). Flag-tagged ER $\alpha\Delta$ AD was immunoprecipitated using anti-Flag M2 antibody. The ubiquitination status of ER $\alpha\Delta$ AD was analyzed by Western blotting using anti-ubiquitin antibody.

mediated through ubiquitination. In the absence of MG132, we detected almost no or little ubiquitination of ER α AD in the presence and absence of estrogen (Figure 1D, lanes 2 and 4). However, in the presence of MG132, we observed smeary bands of ubiquitin-conjugated ER α AD products in the absence of estrogen (Figure 1D, lane 3). These results indicate that while ER α AD shows no ligand-dependent ubiquitination, unliganded ER α AD is still degraded through ubiquitin-proteasome pathways. According to these results, there are possibly two independent ubiquitination pathways for ER α .

Unliganded ER α associates with a protein complex containing CHIP

We then investigated the region responsible for the degradation of unliganded ER α . The protein level of truncated ER α was examined by Western blotting in the presence or absence of estrogen. As shown in Figure 2A, all of the deletion mutants containing the E domain accumulated with estrogen treatment. MG132 treatment increased the levels of these mutants, indicating that they were degraded through proteasome (Figure 2A, lower panel). These results suggest that the region responsible for the degradation of unliganded ER α is located within ER α LBD. From these results, we speculated that an E3 ubiquitin ligase specifically binds and conjugates ubiquitin to the unliganded ER α LBD. We therefore attempted to identify the putative ubiquitin ligase for unliganded ER α . A HeLa cell extract-derived fraction was incubated with glutathione-S-transferase (GST)-fused ER α LBD in the presence or absence of estrogen. Proteins that interacted with ER α LBD were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and silver stained (Figure 2B). To identify the proteins that selectively bound to unliganded ER α LBD, we performed peptide mass fingerprinting, and revealed that the 35 kDa protein eluted from the unliganded ER α LBD column consisted of CHIP (Figure 2B). The result obtained from peptide mass fingerprinting was confirmed by Western blotting using a specific antibody against CHIP (Figure 2B, lower panel).

CHIP is known to possess E3 ubiquitin-ligase activity mediated by its carboxy-terminal U-box domain and has the ability to bind to chaperones Hsp/Hsc70 by means of its

tetratricopeptide repeat (TPR) domain (Scheufler *et al*, 2000; Connell *et al*, 2001; Imai *et al*, 2002). Mass spectrometric analysis also identified chaperone proteins Hsp/Hsc70 (Figure 2B), indicating that CHIP binds unliganded ER α LBD as a protein complex containing Hsp/Hsc70. Thus, we examined the interaction between ER α and CHIP/Hsp/Hsc70 complex using a co-immunoprecipitation method. As shown in Figure 2C, CHIP is selectively co-immunoprecipitated with

Figure 2 The unliganded ER α associated with a protein complex containing CHIP and Hsc/Hsp70. **(A)** The E region of ER α was sufficient for the degradation of unliganded ER α . Indicated Flag-tagged ER α deletion mutants (500 ng) were transfected into 293 cells. These cells were cultured in the presence or absence of estrogen (10^{-8} M) (upper panel) or MG132 (10^{-6} M) (lower panel). To evaluate the protein level of ER α mutants, Western blot analysis was performed using anti-Flag M2 antibody. **(B)** Purification and identification of ER α LBD-interacting proteins. Extracts prepared from HeLa S3 cells were incubated with immobilized GST-ER α LBD in the presence or absence of estrogen (10^{-6} M). ER α -interacting proteins were eluted from the GST-ER α LBD column by *N*-lauroyl sarkosin and subjected to SDS-PAGE followed by silver staining. The fractions eluted from unliganded GST-ER α LBD column (lane 1) and liganded GST-ER α LBD column (lane 2) are shown. Proteins eluted from both columns were examined by mass spectrometry. *Hsc70. **(C)** Interaction between unliganded ER α and CHIP *in vivo*. MCF-7 cells were lysed and subjected to immunoprecipitation using either anti-CHIP or anti-ER α antibody in the presence or absence of indicated ligands (estrogen (10^{-8} M); OHT: 4-hydroxy-tamoxifen (10^{-6} M); ICI: ICI182,780 (10^{-7} M)). The precipitates were Western blotted with antibodies for CHIP, ER α and Hsc70. MCF-7 whole-cell extract is shown in lane 1 (WCE).

unliganded ER α and Hsc70. Cell treatment with either 4-hydroxytamoxifen (OHT), a partial antagonist of ER α , or ICI182,780 (ICI), a pure antagonist of ER α , abrogated the binding between ER α and CHIP. CHIP was also detected in the immunoprecipitation performed with an anti-ER α antibody in the absence of ligands, confirming the interaction between ER α and CHIP *in vivo*. The same results were obtained in the human endometrial adenocarcinoma cell line Ishikawa (data not shown).

To better characterize and identify other components of the CHIP-Hsc70 complex, we generated HeLa cell lines stably expressing Flag-HA double-tagged CHIP. The protein complex containing CHIP was precipitated and separated by SDS-PAGE. Protein identification of the purified proteins by mass spectrometric analysis identified KIAA0678, Hsp90, Hsc70, Hsp70, Hsp40 and CHIP (Figure 3A). The protein components of the CHIP complex were confirmed by Western blotting using specific antibodies. Hsp90, Hsc70, Hsp70, Hsp40 and BAG-1 in the CHIP complex are shared with the chaperone components, whereas other chaperone

components, Hip, Hop and p23, were undetectable by Western blot analysis (Figure 3B). To investigate whether this protein complex binds to unliganded ER α , Flag-tagged ER α expressed in 293 cells was immunoprecipitated using anti-Flag monoclonal antibody. As shown in Figure 3C, all of the components detected in the CHIP complex by Western blotting existed in the precipitant (Figure 3C, left panel). Next, to investigate whether this protein complex has the same composition in physiological conditions, ER α was immunoprecipitated from MCF-7 cells using a specific antibody for ER α . In the absence of estrogen, the protein complex purified from MCF-7 contained the same components as the complex in 293 cells (Figure 3C, right panel), suggesting that this protein complex exists in the physiological conditions.

CHIP ubiquitinates and degrades unliganded ER α

To test whether CHIP is involved in the ubiquitination and degradation of unliganded ER α , either ER α or ER α Δ AD was transfected into 293 cells with or without CHIP. Western blot analysis revealed that, in the absence of estrogen, the steady-state levels of ER α and ER α Δ AD were decreased when CHIP was expressed (Figure 4A; 293, lanes 3 and 5). In contrast, in the presence of estrogen, the expression of CHIP exhibited little or no effect on the protein level of ER α and ER α Δ AD (Figure 4A; 293, lanes 4 and 6). Endogenous ER α in MCF-7 cells was also decreased by CHIP expression (Figure 4A; MCF-7). Cell treatment with MG132 or lactacystin blocked CHIP-dependent ER α degradation, indicating that the degradation is mediated through proteasome pathways (Figure 4A, lower panel). We further determined the CHIP function by developing MCF-7 cells in which endogenous CHIP expression was suppressed by the introduction of a small interfering RNA (siRNA) complementary to sequences present in the CHIP mRNA. The introduction of the siRNA vector into MCF-7 cells resulted in the suppression of CHIP mRNA (data not shown) and protein expression, and the accumulation of ER α protein (Figure 4B). In contrast, a control vector failed to alter the CHIP or ER α protein level. In addition, either OHT or ICI treatment abrogated CHIP-induced ER α degradation (Figure 4C). Considering the observation that OHT- or ICI-bound ER α showed no interaction with CHIP, it is suggested that the degradation requires binding between ER α and CHIP.

To confirm that CHIP enhances unliganded ER α degradation, pulse-chase experiments were performed. In the absence of CHIP, the half-life of unliganded ER α exceeded 12 h (Figure 4D; 293), whereas, in the presence of CHIP, the turnover of unliganded ER α increased and exhibited a half-life of approximately 6 h (Figure 4D; 293). The half-life of estrogen-bound ER α was not changed by the expression of CHIP (data not shown). In MCF-7 cells, CHIP also enhanced the turnover of endogenous ER α in the absence of estrogen (Figure 4D; MCF-7). To test the specificity of this effect, we created constructs in which the TPR and U-box domains of CHIP were deleted (Δ TPR and Δ Ubox). CHIP binds to Hsp/Hsc70 by means of its TPR motif, while also displaying E3 ubiquitin-ligase activity mediated by its U-box domain. Although the expression of these proteins was similar to that of wild-type CHIP (data not shown), the deletion of either of these domains abolished the effects of CHIP on ER α or ER α Δ AD protein level (Figure 5A). The requirement of a TPR motif indicates that CHIP may need to interact with Hsc70 to promote ER α degradation. Functional requirement

Figure 3 Purification and identification of a protein complex containing CHIP. (A, B) HeLa S3 cells (Mock) or HeLa S3 cells constitutively expressing Flag/HA double-tagged CHIP (Flag/HA-CHIP) were subjected to sequential immunoprecipitation using anti-Flag M2 and anti-HA antibody as described in Materials and methods. The purified fractions were subjected to SDS-PAGE followed by silver staining (A). Proteins eluted from these columns were examined by mass spectrometry (A) and Western blotting (B). Total HeLa cell extract is shown in lane 1 (WCE) (B). (C) Unliganded ER α interacted with a protein complex containing chaperones and CHIP. Flag-ER α -transfected 293 cells (ER α), untransfected cells (Mock) or MCF-7 cells were subjected to immunoprecipitation using either anti-Flag M2 (left panel) or anti-ER α (right panel) antibody and then Western blotted using indicated antibodies. The whole-cell extract is shown in lane 1 (WCE).

of the U-box implies that CHIP regulates ER α ubiquitination. In order to validate this model, we evaluated the presence of Hsp/Hsc70 and ER α in complexes containing CHIPATPR or CHIPAUbox. As shown in Figure 5B, CHIPATPR did not have the ability to form a complex with Hsc70 and ER α , indicating that Hsc70 mediates the interaction between ER α and CHIP. Finally, we tested whether CHIP enhances ER α turnover through ubiquitination. When ER α was coexpressed with CHIP, we observed the appearance of smeary bands of ubiquitin-conjugated ER α products (Figure 5C, lanes 3 and 5). In the presence of estrogen, CHIP did not enhance the

conjugation of ubiquitin to ER α (Figure 5C, lanes 2 and 4). Overall, these observations indicate that the ubiquitination and degradation of unliganded ER α is mediated by a protein complex containing CHIP ubiquitin ligase.

CHIP preferentially recognizes and degrades misfolded ER α

To investigate the effect of CHIP on the transcriptional activity of ER α , a luciferase assay was performed as shown in Figure 6A. While the protein level of ER α was reduced by the expression of CHIP (Figure 6B, upper panel), the transcriptional activity of ER α was slightly enhanced by CHIP expression (Figure 6B, lower panel, compare lane 2 with lanes 5 and 8). Therefore, we next estimated the level of transcriptional activity per ER α protein amount. When ER α was coexpressed with CHIP, the level of transcriptional activity per ER α protein was about two-fold higher than ER α alone (Figure 6B, lower panel, compare lane 3 with lanes 6 and 9).

Our results show that CHIP binds to unliganded but not to liganded ER α . In addition, CHIP was localized mainly in the cytoplasm (Figure 6C). From these observations, it is difficult to believe that CHIP acts as a coactivator for ER α in the nucleus. Furthermore, ER α (HE82), which has three amino-acid substitutions in the DNA-binding region (C domain) in ER α and has almost no ability to bind DNA (Mader *et al*, 1989), was also degraded by CHIP, suggesting that the CHIP-dependent degradation of ER α does not require DNA binding. From these results and previous reports (Hohfeld *et al*, 2001; Meacham *et al*, 2001; Murata *et al*, 2001; Goldberg, 2003), we hypothesized that CHIP preferentially ubiquitinates misfolded ER α proteins to eliminate them. CHIP expression may selectively reduce the protein level of unfolded or misfolded ER α , which has less activity than the normal form. Consequently, CHIP could enhance the level of transcriptional activity per ER α protein.

To test this hypothesis, amino-acid substitutions were introduced into ER α to induce protein misfolding. In the absence of ligands, ER α (V364E) (McInerney *et al*, 1996)

Figure 4 CHIP ubiquitinated and degraded unliganded ER α . (A) CHIP facilitated the degradation of unliganded ER α . HA-tagged CHIP (250 ng) was cotransfected into 293 or MCF-7 cells with or without ER α or ER α ΔAD (500 ng) and in the absence or presence of estrogen (10^{-8} M), MG132 or lactacystin (10^{-6} M). The protein level of ER α was examined by Western blotting using anti-ER α antibody. (B) siRNA-mediated suppression of endogenous CHIP. The plasmid containing siRNA specific for CHIP or control vector was introduced into MCF-7 cells. Transfected cells were selected by puromycin. Protein levels of CHIP and ER α were assessed by immunoblotting of whole-cell lysate with the specific antibodies as indicated. (C) CHIP did not alter the steady-state level of ER α in the presence of OHT or ICI. Either ER α or ER α ΔAD (500 ng) was cotransfected into 293 cells with or without HA-CHIP (250 ng) in the absence or presence of the indicated ligands. The protein level of ER α was examined by Western blotting using specific antibodies for ER α . (D) Pulse-chase assay. 293 cells transfected with CHIP (250 ng) and ER α (500 ng) or MCF-7 cells transfected with CHIP (2 μ g) were pulse-labeled with [35 S]methionine and then chased for the indicated times in media containing unlabeled methionine. 35 S-labeled ER α in anti-ER α immunoprecipitate was quantified by phosphorimaging, and the levels in control cells (closed circle) and CHIP-expressing cells (open circle) were plotted relative to the amount present at time 0.

and ER α (C447A) (Reese and Katzenellenbogen, 1992), both of which have an amino-acid substitution in the LBD and exhibit temperature sensitivity, were unstable and degraded faster than wild-type protein at a nonpermissive temperature (37°C). Wild-type ER α also degraded to the same extent as temperature-sensitive mutants when cells were cultured under thermally stressed conditions (cells were cultured at 42°C for 30 min) (Figure 6D, upper panel, compare lane 1

with lanes 2, 3 and 6). In contrast, ER α (L540Q) (Ince *et al*, 1995) and ER α Δ AD, which have either an amino-acid substitution or truncation in the flexible Helix 12 region, exhibited the same stability as wild type at 37°C (Figure 6D, upper panel, compare lane 1 with lanes 4 and 5). Under a permissive temperature (30°C), the protein stability of ER α (V364E) and ER α (C447A) was comparable with that of the wild type (Figure 6D, lower panel).

In a luciferase assay, these four mutated ER α proteins showed a loss or reduction of transcriptional activity compared to the wild type (Figure 6E, lane 5), and they were able to suppress wild-type activity when coexpressed with wild-type ER α (Figure 6E, lane 8). CHIP did not enhance the ER α activity suppressed by ER α (L540Q) or ER α Δ AD; however, transcriptional activity suppressed by ER α (V364E) or ER α (C447A) was recovered by CHIP expression (Figure 6E, lanes 9 and 10). These results suggest that CHIP may preferentially ubiquitinate ER α (V364E) and ER α (C447A) to degrade these mutants.

If CHIP is directly involved in the hydrolysis of abnormal or mutant forms of ER α , then it should be able to form specific complexes with mutated or misfolded ER α . ER α or mutated forms of ER α were immunoprecipitated from transfected cells and the presence of CHIP and chaperone proteins was detected using specific antibodies. At a permissive temperature (30°C), the amount of CHIP in the precipitate pellets with ER α (V364E) or ER α (C447A) was almost the same in precipitates with the wild type (Figure 7A, right panel). However, at a nonpermissive temperature (37°C), CHIP and BAG-1, a co-chaperone that binds to both Hsc70 and the proteasome, preferentially co-immunoprecipitated with ER α (V364E) and ER α (C447A), while the amount of other chaperone components in precipitants was unchanged (Figure 7A, left panel). In addition, thermally stressed conditions (42°C for 30 min) also increased the CHIP and BAG-1 levels in the precipitated pellet (Figure 7A, left panel, lane 6). Consistent with the results obtained from the degradation and interaction experiments, the polyubiquitination of the temperature-sensitive mutants or thermally denatured ER α was enhanced at nonpermissive temperature (Figure 7B, compare left panel with right panel).

Liganded but not unliganded ER α degradation is observed in CHIP $^{-/-}$ cells

To firmly establish the importance of the observation of CHIP-dependent ER α degradation, we isolated mouse embryonic

Figure 5 CHIP-dependent ubiquitination and degradation of ER α required its TPR and U-box domain. (A) Both the TPR and U-box domain in CHIP were necessary for ER α degradation. CHIP, CHIPΔTPR or CHIPΔUbox (250 ng) was transfected into 293 cells with or without ER α or ER α Δ AD (500 ng). Protein levels of ER α and ER α Δ AD were examined by Western blotting using anti-ER α antibody. (B) The TPR domain of CHIP is necessary for binding to Hsc70 and ER α . HA-tagged CHIP or CHIP mutants were expressed in 293 cells and immunoprecipitated with anti-HA antibody in the absence of estrogen. Precipitates were Western blotted with antibodies for CHIP, ER α and Hsc70. (C) CHIP induced the ubiquitination of unliganded ER α . Flag-tagged ER α (500 ng) was transfected into 293 cells with or without CHIP (250 ng) or CHIPΔUbox (250 ng) in the presence or absence of estrogen (10^{-8} M). Flag-tagged ER α was immunoprecipitated using anti-Flag M2 antibody. The ubiquitination status of ER α was analyzed by Western blotting using anti-ubiquitin antibody.

fibroblast (MEF) cells from either CHIP $^{-/-}$, CHIP $+/+$ mice or wild-type littermates, CHIP $+/+$, and determined the protein level of ER α . To induce misfolding of ER α protein, these cells were cultured under thermally stressed conditions. In the absence of estrogen, the thermally stress conditions reduced ER α levels in both CHIP $+/+$ and CHIP $+/+$ cells but not in CHIP $^{-/-}$ cells (Figure 8A, lanes 4–6). MG132 induced the accumulation of ER α in CHIP $+/+$ and CHIP $+/+$ cells, indicating that ER α was degraded through proteasome pathways in these cells. These observations provide

further support for a model in which CHIP preferentially binds misfolded ER α proteins and degrades them to maintain the quality of ER α protein in cells. Co-immunoprecipitation experiments showed the existence of ER α /Hsc70/CHIP complex in CHIP $+/+$ cells but not in CHIP $^{-/-}$ cells (Figure 8B). Furthermore, estrogen treatment induced ER α degradation in CHIP $^{-/-}$ cells to the same extent as in CHIP $+/+$ cells (Figure 8C), suggesting that CHIP is not involved in estrogen-dependent degradation, and supporting the idea that there are two independent ubiquitin–proteasome pathways for ER α (Figure 8D).

Discussion

Estrogen receptor α is regulated by two independent ubiquitin–proteasome pathways

Several studies have mentioned that the AD core region of ER α is essential not only for transactivation but also for estrogen-dependent ER α degradation (Lonard *et al*, 2000). These reports are in good agreement with our result that ER α Δ AD, which has no AD core region, does not show estrogen-dependent degradation. Interestingly, however, MG132 had no effect on ligand-bound ER α Δ AD; the steady-state level of ER α Δ AD in the absence of estrogen is accumulated in the presence of MG132. These results indicate that unliganded ER α Δ AD is still degraded through proteasome pathways. According to these observations, it is possible that the degradation pathway for the unliganded receptor differs from that for liganded. ER α Δ AD might be able to recruit a

Figure 6 CHIP preferentially recognized and degraded misfolded ER α . (A) The time schedule for luciferase assay and Western blot analysis. 293 cells were transfected with indicated plasmids. At 48 h after transfection, cells were treated with estrogen (10^{-8} M) for an additional 12 h and harvested for luciferase assay and Western blotting. (B) The level of transcriptional activity per ER α protein amount was enhanced by CHIP. Upper panel: The steady-state level of ER α or ER α (HE82) was reduced by the expression of CHIP but not by CHIP Δ Ubox. Lower panel: Transcriptional activity of ER α was slightly enhanced by CHIP. ER α (100 ng) and either CHIP or CHIP Δ Ubox (100 ng) were cotransfected into 293 cells with ERE-TATA-Luc (100 ng) and pRSV β GAL (100 ng), and cell extracts were used in a luciferase assay. The protein amount of ER α was quantified by phosphoimaging. The levels of transcriptional activity per ER α protein amount were plotted relative to the level in control cells. (C) Immunocytochemistry of CHIP and ER α . 293 cells were transiently transfected with HA-tagged CHIP and ER α . The mounted cells were examined by immunofluorescence microscopy as described in Materials and methods. Green represents immunofluorescence for HA-CHIP and red ER α . The distribution of CHIP in a cell body is shown in panel a, and panel b shows the distribution of ER α . Panel c shows the merge images of panels a and b. (D) Temperature-sensitive mutants of ER α degraded faster than wild-type ER α in the absence of ligands. ER α (V364E), ER α (C447A), both of which are temperature sensitive, and ER α (L540Q) were generated by amino-acid substitutions of wild-type ER α . Indicated ER α or ER α mutants (500 ng) were transfected into 293 cells in the presence or absence of estrogen (10^{-8} M) and MG132 (10^{-6} M) at 30°C (permissive temperature; lower panel), 37°C (normal/nonpermissive temperature; upper panel) or under thermally stressed conditions (42°C for 30 min; upper panel). Protein levels of ER α or mutants were analyzed by Western blotting using anti-ER α antibody. (E) CHIP recovered the transcriptional activity of ER α suppressed by coexpression of ER α mutants. ER α (100 ng), ERE-TATA-Luc (100 ng) and pRSV β GAL (100 ng) were cotransfected into 293 cells with or without either ER α (V364E), ER α (C447A), ER α (L540Q), ER α Δ AD (100 ng) or CHIP (100 ng), and cell extracts were used in a luciferase assay.

Figure 7 The misfolding of ER α induced the recruitment of CHIP and BAG-1 to the complex. (A) CHIP and BAG-1 preferentially recognized and bound misfolded ER α . Flag-tagged ER α , ER α (V364E) or ER α (C447A) (100 ng) was transfected into 293 cells. These cells were cultured with MG132 (10^{-6} M) at 30°C (permissive temperature; right panel), 37°C (normal/nonpermissive temperature; left panel) or under thermally stressed conditions (42°C for 30 min; left panel). Extracts prepared from these cells (lanes 1–6) or untransfected cells (Mock) were subjected to immunoprecipitation using anti-Flag M2 antibody and then Western blotted using antibodies as indicated. The whole-cell extract is shown in lane 1 (WCE). (B) The ubiquitination status of the temperature-sensitive mutants or heat-shocked ER α was enhanced. Flag-tagged ER α , ER α (V364E) or ER α (C447A) (500 ng) was transfected into 293 cells. These cells were cultured with MG132 (10^{-6} M) at 30°C (right panel), 37°C (left panel) or under thermally stressed conditions (42°C for 30 min; left panel). Extracts prepared from these cells (lanes 2–5) or untransfected cells (Mock) were subjected to immunoprecipitation using anti-Flag M2 antibody. The ubiquitination status of ER α and mutants was analyzed by Western blotting using anti-ubiquitin antibody.

degradation machinery for the unliganded receptor but not for the liganded. Otherwise, there may be a change in the conformation of the receptor, which would protect the receptor from degradation. Reid *et al* (2003) also demonstrated that unliganded ER α is subject to proteasome-mediated turnover, which is mechanistically different from the turnover of liganded ER α .

Several lines of evidence indicate that estrogen, progesterone and glucocorticoid receptors (GRs) are degraded in the presence of their cognate ligands (Nawaz *et al*, 1999a; Wallace and Cidlowski, 2001). However, this is contrasted with observations of androgen and vitamin D receptors, which are accumulated in the presence of their agonist

Figure 8 Liganded but not unliganded ER α degradation was observed in CHIP $^{-/-}$ MEF cells. (A) Thermally induced degradation of ER α was not observed in CHIP $^{-/-}$ cells. MEF cells were isolated from CHIP $^{-/-}$, CHIP $^{+/-}$ mice and wild-type littermates (CHIP $^{+/+}$). MEF cells were cultured under normal conditions (37°C) or thermally stressed conditions (42°C for 30 min) without estrogen. Extracts prepared from the MEF cells were subjected to Western blotting using the indicated antibody. (B) CHIP $^{+/+}$ or CHIP $^{-/-}$ cells were lysed and subjected to immunoprecipitation using anti-ER α antibody in the absence of estrogen. Precipitates were Western blotted with antibodies for ER α , Hsc70 and CHIP. (C) Estrogen induced degradation of ER α in CHIP $^{-/-}$ cells. MEF cells were cultured in the presence or absence of estrogen (10^{-8} M), and cell extracts prepared from these cells were subjected to Western blotting using anti-ER α antibody. (D) ER α degradation may be regulated by two independent ubiquitin–proteasome pathways.

ligands (Li *et al*, 1999). From our results, these inconsistent observations might be explained by the balance between the two degradation pathways in the cells. When the degradation pathway for unliganded receptors is more active than that for liganded receptors, these receptors would stabilize in the presence of ligands. In contrast, when the liganded receptor degradation pathway is stronger than the unliganded receptor degradation pathway, the protein level of receptors is down-regulated by ligand treatment.

CHIP containing a protein complex specifically binds and ubiquitinates unliganded estrogen receptor

To address the mechanism of the ubiquitination and degradation of unliganded ER α , we purified proteins using GST-fused ER α LBD, and identified CHIP, which specifically bound to unliganded ER α LBD. Our findings indicate that CHIP binds unliganded ER α as a protein complex containing Hsp90, Hsc70, Hsp70, Hsp40 and BAG-1, all of which are known to possess or assist chaperoning functions, and a Dna J-like protein, KIAA0678. Dna J is a member of the Hsp40 family of molecular chaperones, which regulate the activity of Hsp70s. Dna J-like proteins that contain regions closely resembling a Dna J domain are suggested to regulate the activity of Dna J proteins during protein translocation, assembly and disassembly (Cheetham and Caplan, 1998).

CHIP expression with ER α enhanced the conjugation of ubiquitin to the receptors and stimulated degradation. Receptor ubiquitination and degradation was abrogated when cells were treated with estrogen. These results are in good agreement with the results obtained from binding experiments. Furthermore, OHT and ICI, both of which inhibited the interaction between CHIP and ER α , reduced the CHIP-mediated degradation of ER α . These findings confirmed the idea that unliganded ER α ubiquitination is mediated by CHIP. In immunostaining, CHIP was largely detected in the cytoplasm (Figure 6C). The localization of CHIP was not changed when cells were cultured under heat-stressed conditions (data not shown). According to these results, CHIP-dependent ER α ubiquitination may occur mainly in the cytoplasm. However, we cannot exclude the possibility that a small amount of CHIP is involved in the ubiquitination of ER α in the nucleus.

Recently, CHIP was reported to induce ubiquitination of the GR bound to Hsp90 for proteasomal degradation (Connell *et al*, 2001). While our findings indicate that CHIP selectively binds to unliganded ER α and ubiquitinates it, CHIP-mediated GR degradation is observed in the presence of ligands. Recent reports indicate that in the presence of ligands, nuclear receptors do not remain permanently bound at a promoter, but rather undergo cycles of binding and unbinding (Shang *et al*, 2000; Stenoien *et al*, 2001; Galigniana *et al*, 2004). The cycling of ligand-bound ER α requires proteasomal activity (Reid *et al*, 2003). Together with these reports and our observations, it is possible that the binding of estrogen to ER α induces the dissociation of CHIP and the association of other ubiquitin ligases, which are involved in receptor cycling at a promoter. The ligand-dependent cycling of GR is known to be much faster than that of ER α and both chaperones and proteasomes are thought to be important for GR cycling since the disruption of either leads to alterations in the exchange rate (Galigniana *et al*, 2004). According to these results, it is possible that, while the chaperone complex containing CHIP

mainly resides in the cytoplasm, it may translocate into the nucleus and regulate the cycling of liganded GR.

CHIP is involved in the quality control of estrogen receptor

Since CHIP selectively bound to and ubiquitinated unliganded ER α , CHIP seemed not to be directly involved in transcriptional regulation. Recently, it was shown that CHIP is involved in the ubiquitination of the immature cystic fibrosis transmembrane conductance regulator (CFTR) in the endoplasmic reticulum-associated degradation (ERAD) pathway (Wickner *et al*, 1999; Meacham *et al*, 2001). Based on these findings, it is speculated that CHIP may be a new category of E3 enzyme responsible for the quality control of cellular proteins linked to the function of molecular chaperones. However, there is no experimental evidence to show that CHIP indeed acts as E3 ubiquitin ligase capable of distinguishing the non-native states from native states of target proteins *in vivo*.

In this study, we have shown that temperature-sensitive mutants of ER α preferentially recruited CHIP to ubiquitinate and degrade these receptors under nonpermissive temperatures. In addition, the ubiquitination and degradation of unliganded ER α was enhanced when cells were cultured under thermally stressed conditions. These observations suggest that CHIP preferentially induces the hydrolysis of abnormal or mutant forms. Using MEF cells derived from CHIP $-/-$ or wild-type littermates, we confirmed the importance of the observation of CHIP-mediated unfolded ER α degradation. These observations provide direct *in vivo* evidence that CHIP selectively ubiquitinated thermally denatured ER α . Our observations provide the first *in vivo* evidence that CHIP functions as 'quality-control E3' involved in the selective ubiquitination of target proteins by recognizing the non-native state in a molecular chaperone-assisted manner. Furthermore, estrogen treatment induced the degradation of ER α in CHIP $-/-$ cells to the same extent as in CHIP $+/+$ cells, suggesting that CHIP is not involved in estrogen-dependent degradation, and supporting the idea that there are two independent ubiquitin-proteasome pathways for ER α . Considering that nuclear receptors have conserved LBDs and that some are known to associate with a chaperone complex, our findings raise the possibility that other members of the nuclear receptor family may also be regulated by two independent ubiquitin-proteasome pathways.

Materials and methods

Expression vectors, antibodies, cell culture and transfection

These are available as Supplementary data at *The EMBO Journal* Online.

Co-immunoprecipitation and Western blotting

293 cells were transfected with the indicated plasmids, lysed in TNE (10 mM Tris-HCl (pH 7.8), 1% NP-40, 0.15 M NaCl, 1 mM EDTA, 1 μ M phenylmethylsulfonyl fluoride (PMSF), 1 μ g/ml aprotinin) buffer. Extracted proteins were immunoprecipitated with the antibody-coated protein A/G Sepharose (Amersham) or anti-Flag M2 agarose (Sigma). The bound proteins were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Millipore) and detected with indicated antibodies, and secondary antibodies conjugated with horseradish peroxidase. Specific proteins were detected using enhanced chemiluminescence (ECL) Western blot detection system (Amersham).

Ubiquitination assay

MCF7 and 293 cells, which were transfected with or without Flag-tagged ER α and HA-tagged CHIP, were lysed with radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with COMPLETE protease inhibitor mixture (Roche) and kept for 20 min on ice. The extracts clarified by centrifugation were immunoprecipitated with anti-Flag agarose for 1 h at 4°C. After washing the resin with RIPA buffer, the bound proteins were eluted by incubation for 1 h at 4°C with Flag peptide in RIPA buffer (0.4 mg/ml). Immunoprecipitates were immunoblotted with the indicated antibody.

Protein purification

Immobilized GST-ER α LBD fusion proteins were preincubated for 1 h at 4°C in GST-binding buffer (20 mM Tris-HCl (pH 7.9), 180 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 1 mM DTT) containing BSA (1 mg/ml) with or without estrogen (10^{-6} M). Bead-immobilized proteins were then incubated at 4°C for 6–10 h with HeLa cell extracts in the presence or absence of 10^{-6} M estrogen. After washing with GST buffer (GST-binding buffer with 0.1% NP-40) three times, the beads were further washed with GST buffer containing 0.2% N-lauroyl sarkosine. Proteins bound to ER α were eluted with 15 mM reduced glutathione in elution buffer (50 mM Tris-HCl (pH 8.3), 150 mM KCl, 0.5 mM EDTA, 0.5 mM PMSF, 5 mM NaF, 0.08% NP-40, 0.5 mg/ml BSA, 10% glycerol). For purification of the Flag/HA-CHIP complex, HeLa cells stably expressing Flag/HA-CHIP were extracted with TNE buffer and extracted proteins were incubated with anti-Flag M2 agarose for 2 h at 4°C. After washing the resin with TNE buffer, the bound proteins were eluted by incubation for 1 h at 4°C with Flag peptide in TNE buffer (0.4 mg/ml). For further purification, eluted fractions were incubated with anti-HA agarose for 2 h at 4°C. After washing with TNE buffer, the bound proteins were eluted with a small aliquot of HA peptide in TNE buffer (0.05 mg/ml).

Pulse chase

MCF7 and 293 cells were transfected with or without ER α and CHIP, and 48 h post-transfection, the cells were labeled for 30 min at 37°C with 50 μ Ci [35 S]methionine per ml in methionine-free Dulbecco's modified Eagle's medium (DMEM). The cells were then washed twice and incubated in DMEM containing 10% FBS for the indicated

time periods (chase). At each time point of the chase, cell lysates were immunoprecipitated with anti-ER α antibody. The immunoprecipitates were resolved by SDS-PAGE and visualized by autoradiography. Phosphorimager was used to quantify the metabolically labeled ER α present at each time point.

Immunofluorescence

The 293 cells were grown on poly-L-lysine-coated eight-well chamber culture slides, and transfected with plasmids. At 24 h post-transfection, the cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with Triton buffer (50 mM Tris-HCl (pH 7.5), 0.5% Triton X-100, 150 mM NaCl, 2 mM EDTA) for 15 min. The cells in each well were blocked with PBS containing 1% BSA and 0.5% goat serum for 3 h at 37°C. The cells were incubated with anti-HA and ER α antibody in PBS containing 1% BSA for 2 h at 37°C. After washing with PBS, the cells were incubated with Alexa fluor 488 goat anti-rat IgG and Alexa fluor 594 goat anti-mouse IgG (Molecular Probes) for 1 h at 37°C and washed with PBS. The sample was mounted in VECTASHIELD mounting medium (Vector Labs) and analyzed with Leica TCS SP2 spectral confocal scanning system.

RNAi

MCF7 cells maintained in the DMEM medium containing charcoal-stripped FBS were cotransfected with CHIP siRNA vector or luciferase siRNA vector (control) and pUC19 vector carrying puromycin-resistant gene. At 24 h post-transfection, the transfected cells were changed to the medium containing 1 μ g/ml of puromycin. At 48 h after puromycin selection, the puromycin-resistant cells were harvested and lysed with TNE buffer. The equal amounts of extracted protein were subjected to Western blotting.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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