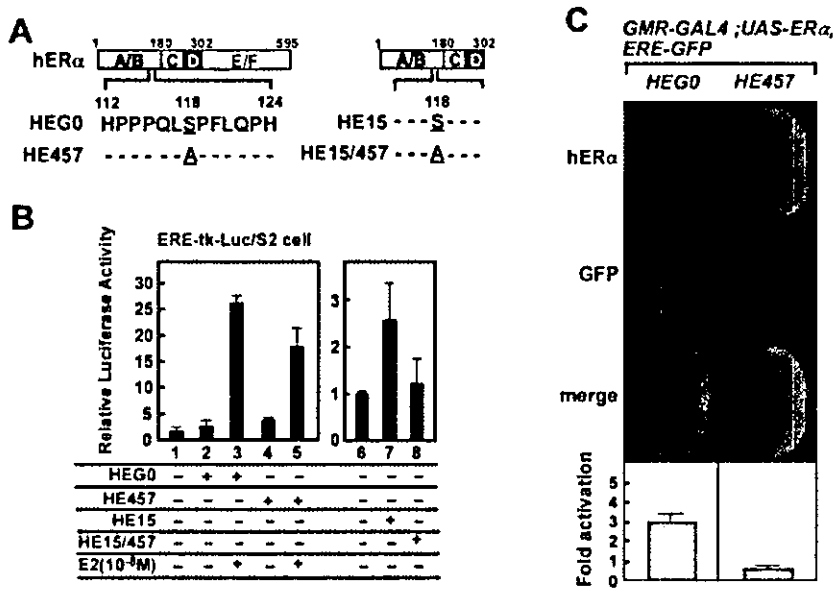

Figure 3 hERα transactivation is regulated by phosphorylation at Ser[118] in *Drosophila*. (A) Schematic representation of hERα mutant constructs. Ser[118] residue is the main phosphorylation site. (B) Transactivation of HEG0 and HE15 mutants in Schneider cells. Schneider cells were transfected with ERE-tk-luc reporter plasmid, Actin-GAL4 plasmid and each hERα mutants, and then incubated with or without 10^{-8} M E2. Luciferase activity data are shown as the average and standard deviation of three independent experiments. (C) Expression (red) and transactivation (green) of hERα mutants in eye imaginal discs. Fold-activation is represented as described (Fig. 1 legend). Genotypes are *GMR-GAL4/SM, UAS-hERα, ERE-GFP/TM3*.

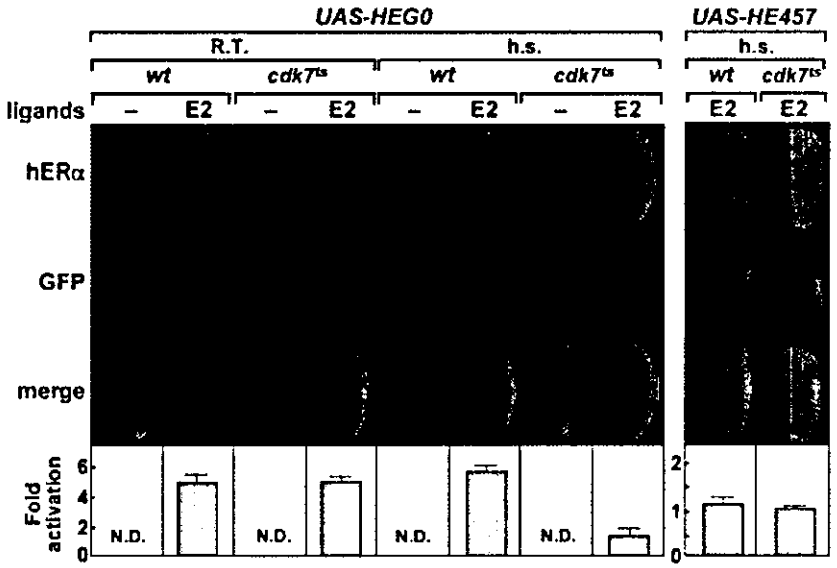

Figure 4 hERα transactivation is enhanced by *Drosophila* Cdk7 through phosphorylation of Ser[118]. hERα expression (red) and transactivation (green) in eye imaginal discs containing single copies of *GMR-GAL4*, *ERE-GFP* and *UAS-hERα* (*HEG0*, *HE457*) with or without heterozygous *cdk7*[+], *cdk7*[ts], the temperature-sensitive *cdk7*[P140S] gene, was introduced into the *Df(1)JB254*-Pw[+]|*snf*[+], *dhd*[+]| (*cdk7*deficient) background. Flies were then incubated at 25 °C (room temperature) or 30 °C (h.s.) for 24 h in medium containing E2. GFP expression levels are represented as described.

HEG0 in *dcdk7*[ts] flies was significantly reduced at 30 °C in comparison with that at room temperature (25 °C). In contrast, HE457 transactivation in *dcdk7*[ts] flies was not affected by exposure to high temperatures (Fig. 4, right panel). These results indicate that Cdk7 potentiated hERα transactivation *in vivo* through Ser[118] phosphorylation.

To further confirm this conclusion, we examined whether hERα Ser[118] is a substrate for dCdk7 *in vitro*. A recombinant GST-fused hERα segment (amino acids 56–180) chimera protein expressed in *E. coli*, and dCdk7 and hCdk7 expressed in 293T cells were used for the *in vitro* phosphorylation assay (Fig. 5A). GST-fused human retinoic acid receptor α1 (hRARα1), a well-characterized substrate for the mammalian Cdk7 (Rochette-Egly *et al.* 1997) was used as a positive control. dCdk7 and hCdk7 were equally capable of phosphorylating hERα and hRARα1. However, the Cdk7 phosphorylation was clearly reduced when the S118A mutant (HE457) was used as a substrate (Fig. 5B).

Activation of the hERα S118A mutant by *Drosophila* AIB1 homologue

Finally, using a fly line with ectopical over-expression of *Drosophila* AIB1 homologue (TAI) in the eye disc, we

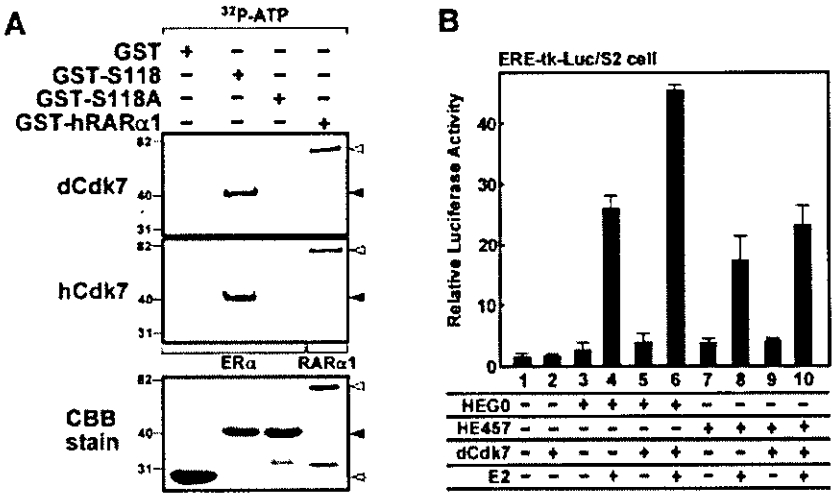

Figure 5 *In vitro* phosphorylation of hERα at Ser[118] by dCdk7. (A) Ten micrograms of GST-fused hERα (amino acids 56–180) and GST-fused hRARα1 were incubated with 9 μg dCdk7 or hCdk7. Phosphorylation and expression of GST-fused hERα amino acids (open arrow head), GST-fused hRARα1 (black arrow head), and GST (grey arrow head) were detected by autoradiography and CBB staining, respectively. (B) Schneider cells were co-transfected with 0.5 μg dCdk7 expression plasmid, 0.5 μg ERE-tk-luc reporter plasmid, 0.2 μg actin-GAL4 plasmid and hERα mutants and then incubated with or without 10^{-8} M E2. Luciferase activity data are shown as the average and standard deviation of three independent experiments.

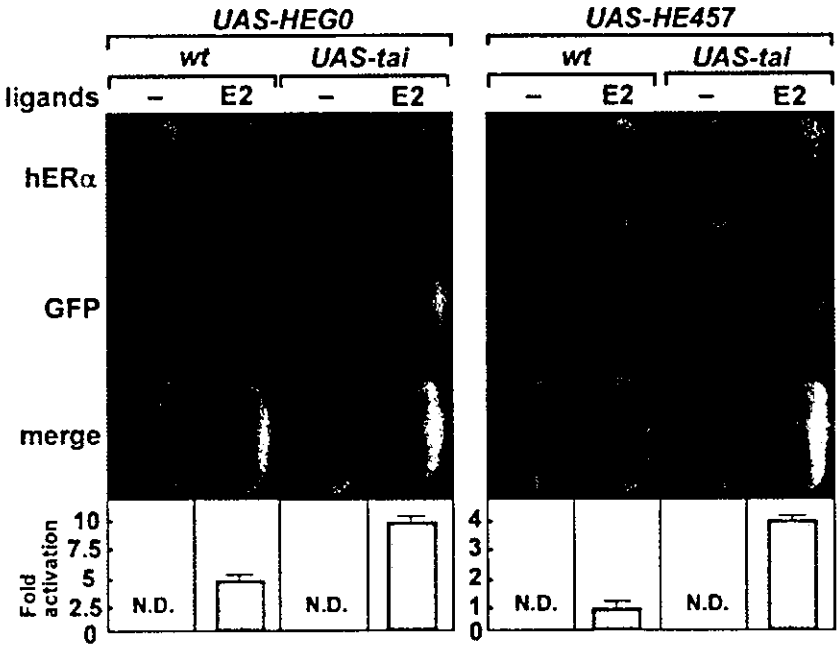

Figure 6 TAI enhancement of hERα transactivation is not dependent on Ser[118] phosphorylation status. hERα expression (red) and transactivation (green) in eye imaginal discs of either HEG0 or HE457 expression lines are shown. TAI is also expressed driven by *GMR-GAL4*.

addressed a question whether enhancement of hERα transactivation by the p160/CBP complex is dependent on the receptor Ser[118] phosphorylation status. Although the hERα S118A mutant was less effective in the ligand-induced transactivation, TAI significantly enhanced transcriptional activity of both the mutant and the wild-type receptor (Fig. 6). This suggests that modulation of the ligand-induced hERα transactivation by the p160/CBP co-activator complex does not depend on the receptor phosphorylation.

Discussion

hERα expressed in *Drosophila* is functional in ligand-induced transactivation

We have previously shown that the human androgen receptor (hAR) ectopically expressed in *Drosophila* tissues was transcriptionally functional and responsive to hAR ligands similar to that in mammalian cultured cells and intact tissues (Takeyama *et al.* 2002). In the present study, we utilized the same approach and demonstrated

that hERα expressed in *Drosophila* was able to activate the ERE reporter gene and respond to hERα agonists and antagonists in the same manner that had been observed in mammalian cells and tissues (McDonnell *et al.* 1995; Metzger *et al.* 1995; Watanabe *et al.* 2001). As hERα transgenic flies appear to be normal in terms of growth and reproduction, without any overt abnormalities, it seems that human steroid hormone receptors do not significantly interfere with endogenous signalling pathways. It can also be inferred that exogenous human steroid receptors do not compete with endogenous NRs at the fly NR-responsive elements in target gene promoters (Talbot *et al.* 1993; McKenna & O'Malley 2002). Therefore, our results provided evidence that transgenic *Drosophila* expressing hERα represent a potent and functionally relevant system in which to evaluate NR synthetic ligands and to genetically identify and characterize novel NR co-regulators.

Pivotal role of Ser^{118} in the hERα ligand-induced transactivation function *in vivo*

Both N-terminal AF-1 and C-terminal AF-2 domains contribute to the hERα ligand-induced transactivation function, with each AF-1 and AF-2 activity dependent on promoter-context and cell type (Kumar *et al.* 1987; Tora *et al.* 1989). The balance between hERα AF-1 and AF-2 is thought to be responsible, at least in part, for the tissue-specific action of selective oestrogen receptor modulators (SERMs) such as tamoxifen (Berry *et al.* 1990; McDonnell *et al.* 1995; Metzger *et al.* 1995; Brzozowski *et al.* 1997; Shiau *et al.* 1998). In particular, the activity of hERα AF-1 is believed to support the oestrogenic actions of SERMs (Endoh *et al.* 1999; Watanabe *et al.* 2001), leading to beneficial actions of SERMs in certain tissues such as the improved bone properties in oestrogen-related pathophysiological states (Shang & Brown 2002). Therefore, while the physiological and pharmacological significance of hERα AF-1 activity has been well addressed, the molecular basis underlying AF-1 function remains to be elucidated in terms of identifying the relevant specific co-regulators and co-regulator complexes (Endoh *et al.* 1999; Watanabe *et al.* 2001). The core activation region of hERα AF-1 has been mapped to the middle of the A/B domain (Kobayashi *et al.* 2000), and a number of *in vitro* studies have indicated that the Ser^{118} residue in this core region appears to play a crucial role and can be phosphorylated by several kinases in response to extracellular signals (Kato *et al.* 1995; Chen *et al.* 2000). Nevertheless, the impact of Ser^{118} phosphorylation *in vivo* remains obscure because of lack of studies involving intact animals. The present findings provide for the first time *in vivo* evidence for the significance of Ser^{118} phosphorylation in the transcriptional activity of the AF-1 domain alone and in the transactivation function of hERα as a whole receptor.

In vivo potentiation of hERα AF-1 through Cdk7-mediated phosphorylation of Ser^{118}

It has been shown that hERα Ser^{118} can be phosphorylated by several kinases (Ali *et al.* 1993; Le *et al.* 1994; Kato *et al.* 1995; Chen *et al.* 2000). Cdk7 has been chosen for the present study as mutant flies with inactive Cdk7 appear to suffer more general defects in gene regulation (Austin & Biggin 1996). We have shown that Cdk7 phosphorylates hERα Ser^{118} *in vivo* and that this phosphorylation enhanced hERα AF-1 activity in normal flies. It has been shown recently that, besides direct receptor phosphorylation, MAPKs are also able to potentiate function of some hERα co-activators, including AIB1, through phosphorylation of the cofactor protein (Font de Mora & Brown 2000). This suggests an additional mechanism for downstream cross-talk between different signalling pathways. Our transgenic *Drosophila* provides an experimental system in which to further study whether MAPKs activated by growth factors or stress-induced signalling pathways can also modulate hERα activity.

Ser^{118} phosphorylation-dependent and -independent co-activators for hERα

The S118A hERα mutant retained ligand responsiveness, albeit with reduced transactivation. Transactivation in the S118A hERα mutant has nevertheless been significantly enhanced by over-expression of TAI, *Drosophila* AIB1 homologue. Therefore, it appears that hERα activity is modulated *in vivo* by both phosphorylation-dependent and phosphorylation-independent co-activators. However, the timing of the recruitment of these co-activators, presumably within co-factor complexes associated with the AF-1 domain, remains unclear. p68/p72 have been identified as hERα AF-1-specific co-activators that physically associate with the hERα AF-1 domain (Endoh *et al.* 1999; Watanabe *et al.* 2001). Significantly, this interaction was clearly not dependent on Ser^{118} phosphorylation. It is not clear, however, whether recruitment of most of known hERα co-activators is dependent on phosphorylation status of the receptor. In this respect, the transgenic *Drosophila* lines that express hERα and its mutants represent a powerful tool for genetic screening of phosphorylation-dependent and -independent co-factors.

Experimental procedures

Transfection and luciferase activity

hERα mutants and dCdk7 expression vectors were constructed using the pCaSpeR vector for expression in Schneider cells. hERα mutants and dCdk7 expression plasmids (0.05 μg) were co-transfected with 0.2 μg actin-GAL4 plasmid and 0.5 μg ERE-tk-luc plasmid, along with 10 ng pRL-CMV-luc plasmid as an internal control. Three hours after transfection, the ligands 10^{-8} M 17β-oestradiol (Sigma, St Louis, MO), 10^{-8} M tamoxifen (Sigma) or 10^{-8} M ICI 182,780 (Tocris Cookson, Ballwin, MO) were added. After 20 h, dual luciferase assays were performed as previously described (Yanagisawa *et al.* 2002).

Generation of transgenic flies and *Drosophila* stocks

For germ-line transformation into *Drosophila*, cDNA encoding hERα mutants and GFP reporter under control of ERE-containing promoter were inserted into pCaSpeR. Transgenic constructs together with pπ25.7wc transposase were microinjected into w^{1118} embryos using a micromanipulator (Leica). Several independent transformant lines were established. To express hERα in *Drosophila* eyes, transgenic lines were crossed with a *GMR-GAL4* line that expressed GAL4 in the retina under the control of the glass multimer reporter. The *tai*^{k05809}, *UAS-tai*, *Df(1)J8254*-Pw^{+}]*snf*$^{+}$, *dhd*$^{+}$] and *cdk7*^{c} mutants were obtained from the Bloomington *Drosophila* Stock Center. The *ncj*^{1} and *GMR-GAL4* line were the generous gifts of Drs S. Ishii and Y. Hiromi, respectively.

Histology

Eye imaginal discs from third instar larvae were dissected and fixed for 20 min in 4% formaldehyde at 25 °C. Eye discs were incubated with primary antibodies HC-20 (Santa Cruz Biotechnology, Santa Cruz, CA) or B10 that recognize the C- and N-terminal regions of hERα, respectively. Cy5-conjugated Affinity Pure donkey anti-rabbit or anti-mouse IgG (Jackson Immuno-Research, West Grove, PA) were used as secondary antibodies for immunofluorescence staining. hERα and GFP expression were detected using a Zeiss Confocal Laser Scanning System 510.

Western blotting

To confirm hERα and GFP expression in *Drosophila*, cell lysates from the heads of adult flies of third instar larvae were separated by 15% SDS–PAGE and detected with anti-ERα antibodies (HC-20 or B10) and anti-GFP antibody (Santa Cruz Biotechnology), and expression levels measured using Adobe Photoshop software facility. Fold-activation of hERα in *Drosophila* was shown as GFP expression signal intensity normalizing with hERα expression signal intensity.

In vitro phosphorylation

293T cells were transfected with FLAG tagged dCdk7 expression plasmid, lysed in lysis buffer, and immunoprecipitated with anti-FLAG affinity gel (Sigma). hCdk7 was obtained from 293T cells by immunoprecipitation with Cdk7 (N-19) antibody (Santa Cruz Biotechnology). dCdk7 or hCdk7 (9 μg) were incubated for 20 min at 30 °C with purified bacterially produced 10 μg of GST-fused hERα (amino acids 56–180 of hERα) and its mutants or GST-fused human retinoic acid receptor α1 (hRARα1) (Rochette-Egly *et al.* 1997), in 50 mM Tris-HCl, 0.5 mM EDTA, 25 mM $MgCl_2$, 1 mM DTT, 20 μM ATP, 0.01 μCi [γ-^{32}P]ATP and 10% glycerol. Phosphorylation of substrates was analysed by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Expression of GST-hERα mutants and GST-hRARα1 were detected by CBB staining.

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

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Recent evidence indicates that the transactivation of estrogen receptor α (ERα) requires estrogen-dependent receptor ubiquitination and degradation. Here we show that estrogen-unbound (unliganded) ERα is also ubiquitinated and degraded through a ubiquitin–proteasome pathway. To investigate this ubiquitin–proteasome pathway, we purified the ubiquitin ligase complex for unliganded ERα and identified a protein complex containing the carboxyl terminus of Hsc70-interacting protein (CHIP). CHIP preferentially bound to misfolded ERα and ubiquitinated it to induce degradation. Ligand binding to the receptor induced the dissociation of CHIP from ERα. In CHIP−/− cells, the degradation of unliganded ERα was abrogated; however, estrogen-induced degradation was observed to the same extent as in CHIP+/+ cells. Our findings suggest that ERα is regulated by two independent ubiquitin–proteasome pathways, which are switched by ligand binding to ERα. 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 ERα and ERβ, 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. ERα 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 ERα-interacting proteins have been described as coactivators or corepressors that modify ERα transcriptional activity (Shang *et al*, 2000; Yanagisawa *et al*, 2002; Metivier *et al*, 2003).

Crystal-structural analysis of ERα 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 ERα, 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 ERα 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 ERα protein levels remain poorly understood.

Here we show that, in the absence of estrogen, ERα 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 ERαLBD, whereas the ubiquitination of the unliganded receptor did not, raised the possibility that the ubiquitin ligase for unliganded ERα might differ from the ligase involved in estrogen-dependent ubiquitination. Therefore, we purified

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the ubiquitin-ligase complex for unliganded ERα 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 ERα 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 ERα 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 ERα is degraded through a ubiquitin–proteasome pathway

As shown in Figure 1A, addition of estrogen to MCF-7 cells reduced the level of ERα protein. The reduction of ERα was inhibited by the proteasome inhibitors MG132 or lactacystin. In the absence of estrogen, MG132 or lactacystin treatment also resulted in ERα accumulation (Figure 1A, lanes 3 and 5), suggesting that not only estrogen-bound ERα but also unliganded ERα is degraded through proteasomes. In ubiquitination assay, ERα 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 ERα is regulated by the same ubiquitin-proteasome pathway. It has been reported that truncated ERα, ERαΔAD, 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 ERαΔAD. ERα and ERαΔAD were transfected into 293 cells and the ERα protein level was examined by Western blot analysis. While the ERα degradation was observed regardless of estrogen treatment, ERαΔAD was stabilized by ligand binding, as it accumulates in response to estrogen. MG132 treatment increases the levels of ERαΔAD in the absence of the ligand but does not affect its estrogen-induced accumulation (Figure 1C). We next tested whether ERαΔAD turnover is

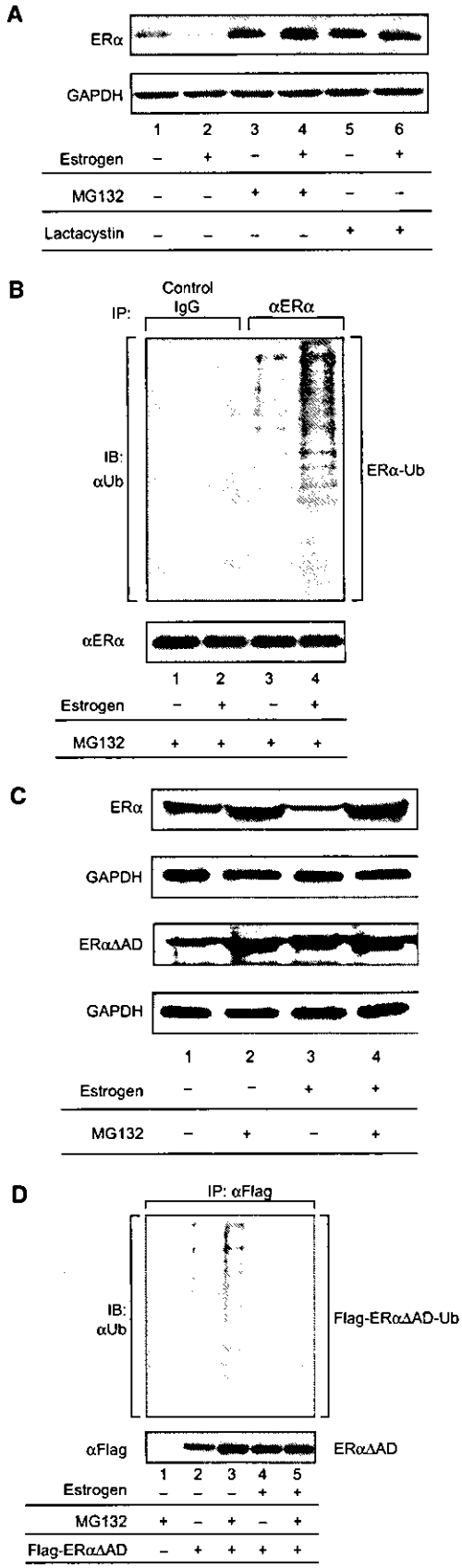

Figure 1 Unliganded ERα was degraded through a ubiquitin–proteasome pathway. (**A**) ERα was degraded in the absence of estrogen. The MCF-7 cells were cultured in the presence or absence of estrogen (10^{-8} M), or the proteasome inhibitor MG132 or lactacystin (10^{-6} M). ERα level was analyzed by Western blotting using anti-ERα monoclonal antibody. (**B**) ERα 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). ERα was immunoprecipitated using anti-ERα antibody. The ubiquitination status of ERα was analyzed by Western blotting using anti-ubiquitin antibody. (**C**) ERαΔAD was selectively degraded in the absence of estrogen. 293 cells were transfected with either ERα or ERαΔAD (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). ERα or ERαΔAD protein levels were analyzed by Western blotting using anti-ERα antibody. (**D**) ERαΔAD was ubiquitinated in the absence of estrogen. Flag-tagged ERαΔAD (500 ng) was transfected into 293 cells in the presence or absence of estrogen (10^{-8} M) or MG132 (10^{-6} M). Flag-tagged ERαΔAD was immunoprecipitated using anti-Flag M2 antibody. The ubiquitination status of ERαΔAD 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 ERαΔAD 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 ERαΔAD products in the absence of estrogen (Figure 1D, lane 3). These results indicate that while ERαΔAD shows no ligand-dependent ubiquitination, unliganded ERαΔAD is still degraded through ubiquitin–proteasome pathways. According to these results, there are possibly two independent ubiquitination pathways for ERα.

Unliganded ERα associates with a protein complex containing CHIP

We then investigated the region responsible for the degradation of unliganded ERα. The protein level of truncated ERα 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 ERα is located within ERαLBD. From these results, we speculated that an E3 ubiquitin ligase specifically binds and conjugates ubiquitin to the unliganded ERαLBD. We therefore attempted to identify the putative ubiquitin ligase for unliganded ERα. A HeLa cell extract-derived fraction was incubated with glutathione-*S*-transferase (GST)-fused ERαLBD in the presence or absence of estrogen. Proteins that interacted with ERαLBD were separated by SDS-polyacrylamide gel electrophoresis (SDS–PAGE) and silver stained (Figure 2B). To identify the proteins that selectively bound to unliganded ERαLBD, we performed peptide mass fingerprinting, and revealed that the 35 kDa protein eluted from the unliganded ERαLBD 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 ERαLBD as a protein complex containing Hsp/Hsc70. Thus, we examined the interaction between ERα and CHIP/Hsp/Hsc70 complex using a co-immunoprecipitation method. As shown in Figure 2C, CHIP is selectively co-immunoprecipitated with

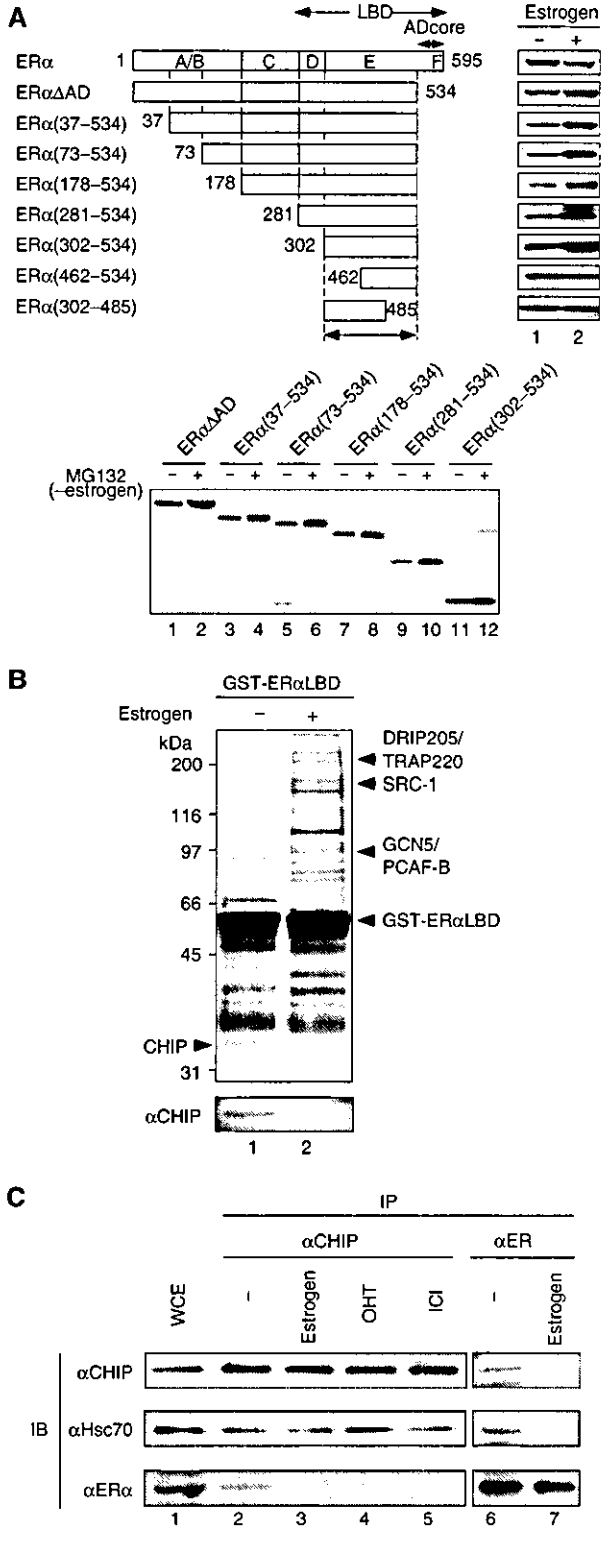

Figure 2 The unliganded ERα associated with a protein complex containing CHIP and Hsc/Hsp70. (**A**) The E region of ERα was sufficient for the degradation of unliganded ERα. Indicated Flag-tagged ERα deletion mutants (500 ng) were transfected into 293 cells. These cells were cultured in the presence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

unliganded ERα and Hsc70. Cell treatment with either 4-hydroxytamoxifen (OHT), a partial antagonist of ERα, or ICI182,780 (ICI), a pure antagonist of ERα, abrogated the binding between ERα and CHIP. CHIP was also detected in the immunoprecipitation performed with an anti-ERα antibody in the absence of ligands, confirming the interaction between ERα 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 ERα, Flag-tagged ERα 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, ERα was immunoprecipitated from MCF-7 cells using a specific antibody for ERα. 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.

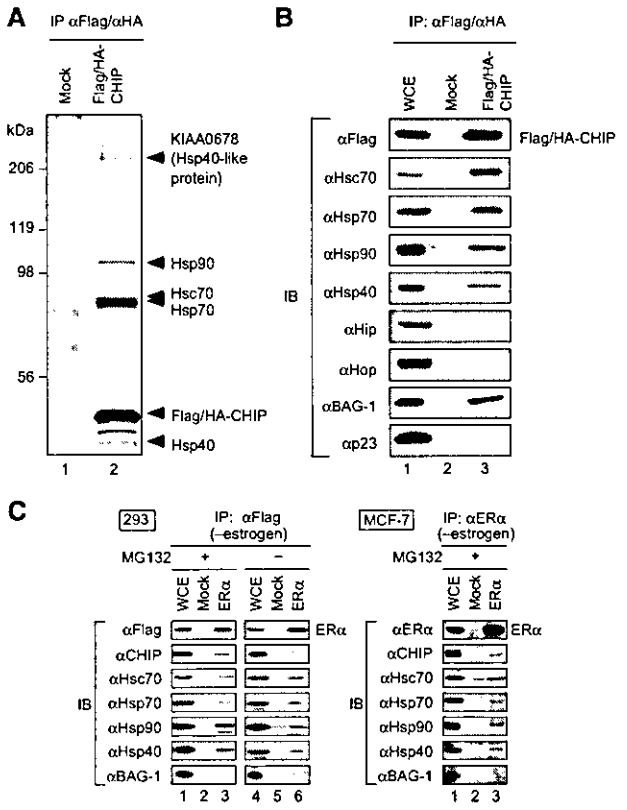

Figure 3 Purification and identification of a protein complex containing CHIP. (**A**, **B**) HeLa S3 cells (Mock) or HeLa S3 cells constitutively expressing Flag/HA double-tagged CHIP (Flag/HA-CHIP) were subjected to sequential immunoprecipitation using anti-Flag M2 and anti-HA antibody as described 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 ERα interacted with a protein complex containing chaperones and CHIP. Flag-ERα-transfected 293 cells (ERα), untransfected cells (Mock) or MCF-7 cells were subjected to immunoprecipitation using either anti-Flag M2 (left panel) or anti-ERα (right panel) antibody and then Western blotted using indicated antibodies. The whole-cell extract is shown in lane 1 (WCE).

CHIP ubiquitinates and degrades unliganded ERα

To test whether CHIP is involved in the ubiquitination and degradation of unliganded ERα, either ERα or ERαΔAD was transfected into 293 cells with or without CHIP. Western blot analysis revealed that, in the absence of estrogen, the steady-state levels of ERα and ERαΔAD 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 ERα and ERαΔAD (Figure 4A; 293, lanes 4 and 6). Endogenous ERα in MCF-7 cells was also decreased by CHIP expression (Figure 4A; MCF-7). Cell treatment with MG132 or lactacystin blocked CHIP-dependent ERα 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 ERα protein (Figure 4B). In contrast, a control vector failed to alter the CHIP or ERα protein level. In addition, either OHT or ICI treatment abrogated CHIP-induced ERα degradation (Figure 4C). Considering the observation that OHT- or ICI-bound ERα showed no interaction with CHIP, it is suggested that the degradation requires binding between ERα and CHIP.

To confirm that CHIP enhances unliganded ERα degradation, pulse-chase experiments were performed. In the absence of CHIP, the half-life of unliganded ERα exceeded 12 h (Figure 4D; 293), whereas, in the presence of CHIP, the turnover of unliganded ERα increased and exhibited a half-life of approximately 6 h (Figure 4D; 293). The half-life of estrogen-bound ERα was not changed by the expression of CHIP (data not shown). In MCF-7 cells, CHIP also enhanced the turnover of endogenous ERα 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 (ΔTPR and ΔUbox). 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 ERα or ERαΔAD protein level (Figure 5A). The requirement of a TPR motif indicates that CHIP may need to interact with Hsc70 to promote ERα degradation. Functional requirement

of the U-box implies that CHIP regulates ERα ubiquitination. In order to validate this model, we evaluated the presence of Hsp/Hsc70 and ERα in complexes containing CHIPΔTPR or CHIPΔUbox. As shown in Figure 5B, CHIPΔTPR did not have the ability to form a complex with Hsc70 and ERα, indicating that Hsc70 mediates the interaction between ERα and CHIP. Finally, we tested whether CHIP enhances ERα turnover through ubiquitination. When ERα was coexpressed with CHIP, we observed the appearance of smeary bands of ubiquitin-conjugated ERα products (Figure 5C, lanes 3 and 5). In the presence of estrogen, CHIP did not enhance the conjugation of ubiquitin to ERα (Figure 5C, lanes 2 and 4). Overall, these observations indicate that the ubiquitination and degradation of unliganded ERα is mediated by a protein complex containing CHIP ubiquitin ligase.

CHIP preferentially recognizes and degrades misfolded ERα

To investigate the effect of CHIP on the transcriptional activity of ERα, a luciferase assay was performed as shown in Figure 6A. While the protein level of ERα was reduced by the expression of CHIP (Figure 6B, upper panel), the transcriptional activity of ERα 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 ERα protein amount. When ERα was coexpressed with CHIP, the level of transcriptional activity per ERα protein was about two-fold higher than ERα 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 ERα. 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 ERα in the nucleus. Furthermore, ERα(HE82), which has three amino-acid substitutions in the DNA-binding region (C domain) in ERα and has almost no ability to bind DNA (Mader *et al*, 1989), was also degraded by CHIP, suggesting that the CHIP-dependent degradation of ERα 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 ERα proteins to eliminate them. CHIP expression may selectively reduce the protein level of unfolded or misfolded ERα, which has less activity than the normal form. Consequently, CHIP could enhance the level of transcriptional activity per ERα protein.

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

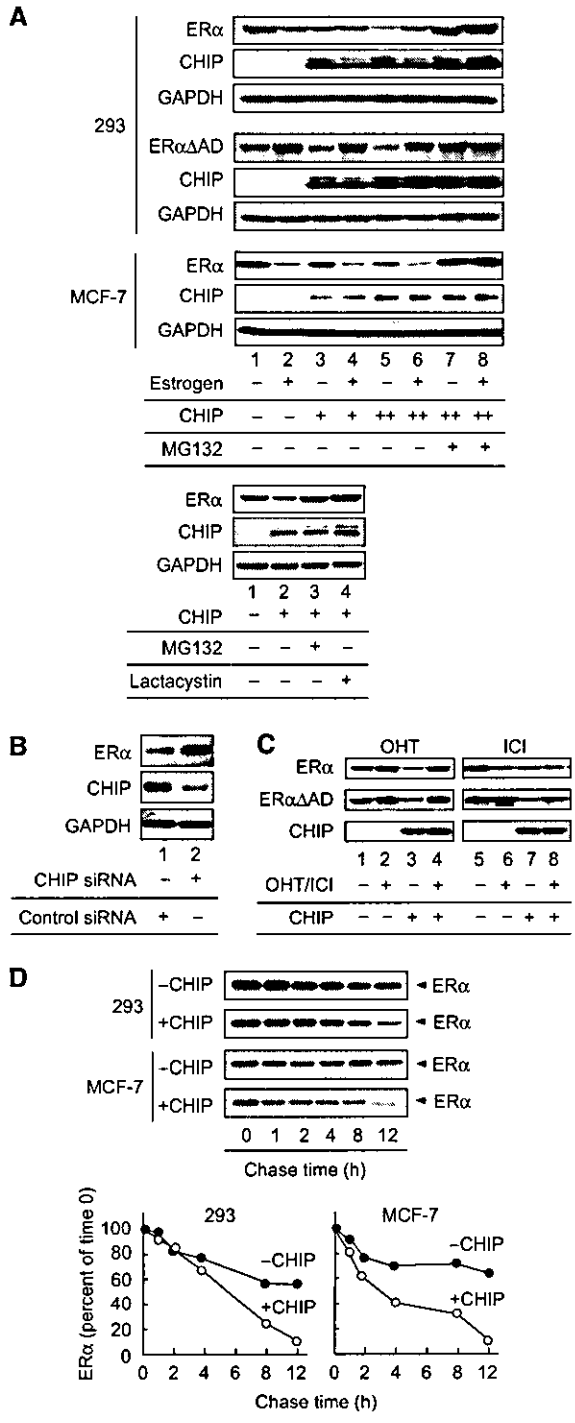

Figure 4 CHIP ubiquitinated and degraded unliganded ERα. **(A)** CHIP facilitated the degradation of unliganded ERα. HA-tagged CHIP (250 ng) was cotransfected into 293 or MCF-7 cells with or without ERα or ERαΔAD (500 ng) and in the absence or presence of estrogen (10^{-8} M), MG132 or lactacystin (10^{-6} M). The protein level of ERα was examined by Western blotting using anti-ERα 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 ERα were assessed by immunoblotting of whole-cell lysate with the specific antibodies as indicated. **(C)** CHIP did not alter the steady-state level of ERα in the presence of OHT or ICI. Either ERα or ERαΔAD (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 ERα was examined by Western blotting using specific antibodies for ERα. **(D)** Pulse-chase assay. 293 cells transfected with CHIP (250 ng) and ERα (500 ng) or MCF-7 cells transfected with CHIP (2 μg) were pulse-labeled with [^{35}S]methionine and then chased for the indicated times in media containing unlabeled methionine. ^{35}S-labeled ERα in anti-ERα immunoprecipitate was quantified by phosphoimaging, 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 ERα(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 ERα 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, ERα(L540Q) (Ince *et al*, 1995) and ERαΔAD, 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 ERα(V364E) and ERα(C447A) was comparable with that of the wild type (Figure 6D, lower panel).

In a luciferase assay, these four mutated ERα 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 ERα (Figure 6E, lane 8). CHIP did not enhance the ERα activity suppressed by ERα(L540Q) or ERαΔAD; however, transcriptional activity suppressed by ERα(V364E) or ERα(C447A) was recovered by CHIP expression (Figure 6E, lanes 9 and 10). These results suggest that CHIP may preferentially ubiquitinate ERα(V364E) and ERα(C447A) to degrade these mutants.

If CHIP is directly involved in the hydrolysis of abnormal or mutant forms of ERα, then it should be able to form specific complexes with mutated or misfolded ERα. ERα or mutated forms of ERα 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 ERα(V364E) or ERα(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 ERα(V364E) and ERα(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 ERα was enhanced at nonpermissive temperature (Figure 7B, compare left panel with right panel).

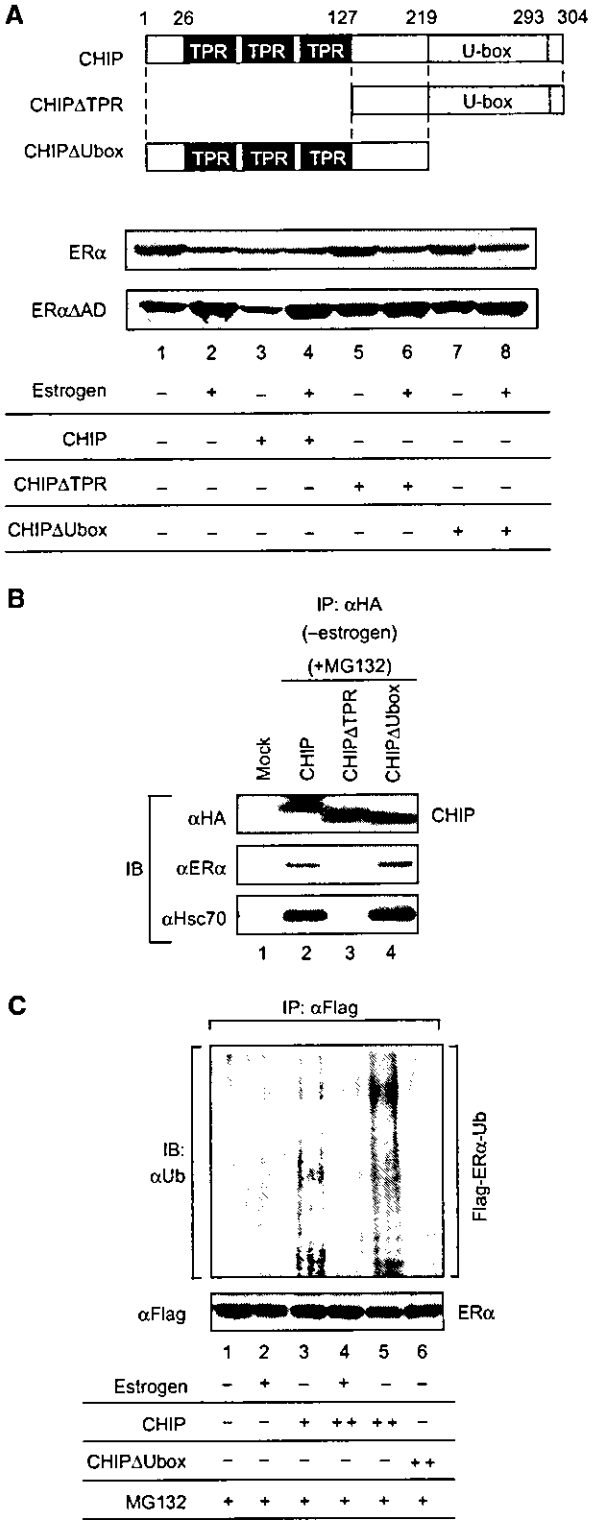

Figure 5 CHIP-dependent ubiquitination and degradation of ERα required its TPR and U-box domain. (**A**) Both the TPR and U-box domain in CHIP were necessary for ERα degradation. CHIP, CHIPΔTPR or CHIPΔUbox (250 ng) was transfected into 293 cells with or without ERα or ERαΔAD (500 ng). Protein levels of ERα and ERαΔAD were examined by Western blotting using anti-ERα antibody. (**B**) The TPR domain of CHIP is necessary for binding to Hsc70 and ERα. 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, ERα and Hsc70. (**C**) CHIP induced the ubiquitination of unliganded ERα. Flag-tagged ERα (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 ERα was immunoprecipitated using anti-Flag M2 antibody. The ubiquitination status of ERα was analyzed by Western blotting using anti-ubiquitin antibody.

Liganded but not unliganded ERα degradation is observed in CHIP−/− cells

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

fibroblast (MEF) cells from either CHIP−/−, CHIP+/− mice or wild-type littermates, CHIP+/+, and determined the protein level of ERα. To induce misfolding of ERα protein, these cells were cultured under thermally stressed conditions. In the absence of estrogen, the thermally stress conditions reduced ERα levels in both CHIP+/+ and CHIP+/− cells but not in CHIP−/− cells (Figure 8A, lanes 4-6). MG132 induced the accumulation of ERα in CHIP+/+ and CHIP+/− cells, indicating that ERα was degraded through proteasome pathways in these cells. These observations provide further support for a model in which CHIP preferentially binds misfolded ERα proteins and degrades them to maintain the quality of ERα protein in cells. Co-immunoprecipitation experiments showed the existence of ERα/Hsc70/CHIP complex in CHIP+/+ cells but not in CHIP−/− cells (Figure 8B). Furthermore, estrogen treatment induced ERα 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 ERα (Figure 8D).

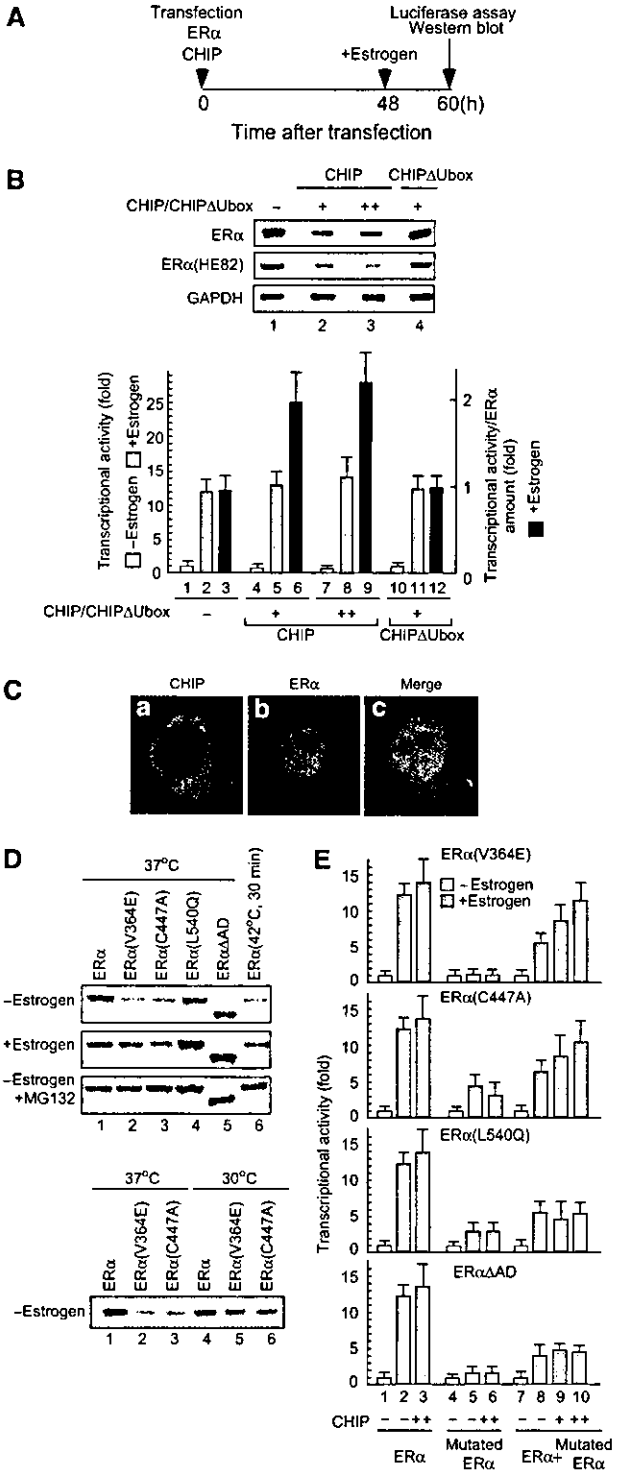

Figure 6 CHIP preferentially recognized and degraded misfolded ERα. **(A)** The time schedule for luciferase assay and Western blot analysis. 293 cells were transfected with indicated plasmids. At 48 h after transfection, cells were treated with estrogen (10^{-8} M) for an additional 12 h and harvested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

Discussion

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

Several studies have mentioned that the AD core region of ERα is essential not only for transactivation but also for estrogen-dependent ERα degradation (Lonard *et al*, 2000). These reports are in good agreement with our result that ERαΔAD, which has no AD core region, does not show estrogen-dependent degradation. Interestingly, however, MG132 had no effect on ligand-bound ERαΔAD; the steady-state level of ERαΔAD in the absence of estrogen is accumulated in the presence of MG132. These results indicate that unliganded ERαΔAD 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. ERαΔAD might be able to recruit a

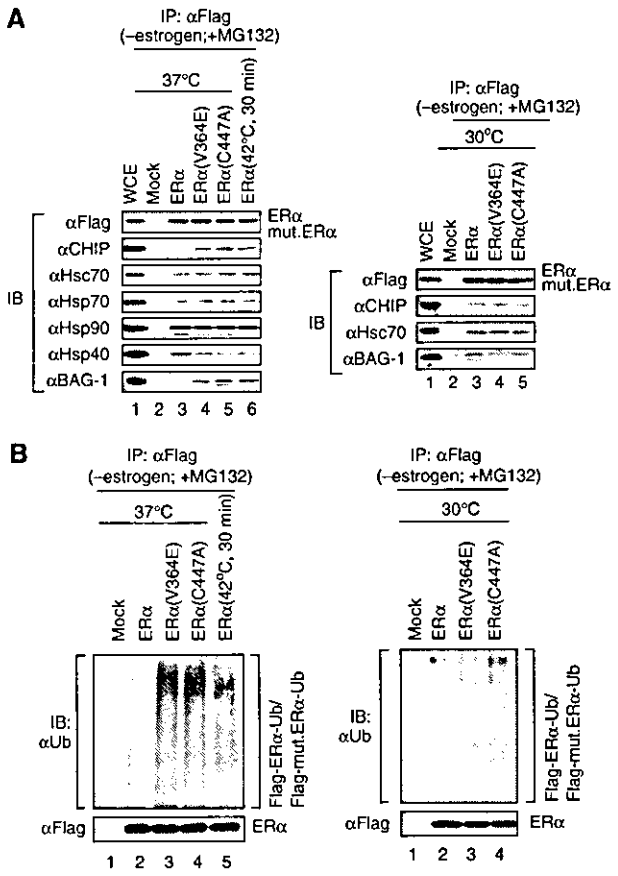

Figure 7 The misfolding of ERα induced the recruitment of CHIP and BAG-1 to the complex. (**A**) CHIP and BAG-1 preferentially recognized and bound misfolded ERα. Flag-tagged ERα, ERα(V364E) or ERα(C447A) (100 ng) was transfected into 293 cells. These cells were cultured with MG132 (10^{-6} M) at 30°C (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 3–6) 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 ERα was enhanced. Flag-tagged ERα, ERα(V364E) or ERα(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 ERα and mutants was analyzed by Western blotting using anti-ubiquitin antibody.

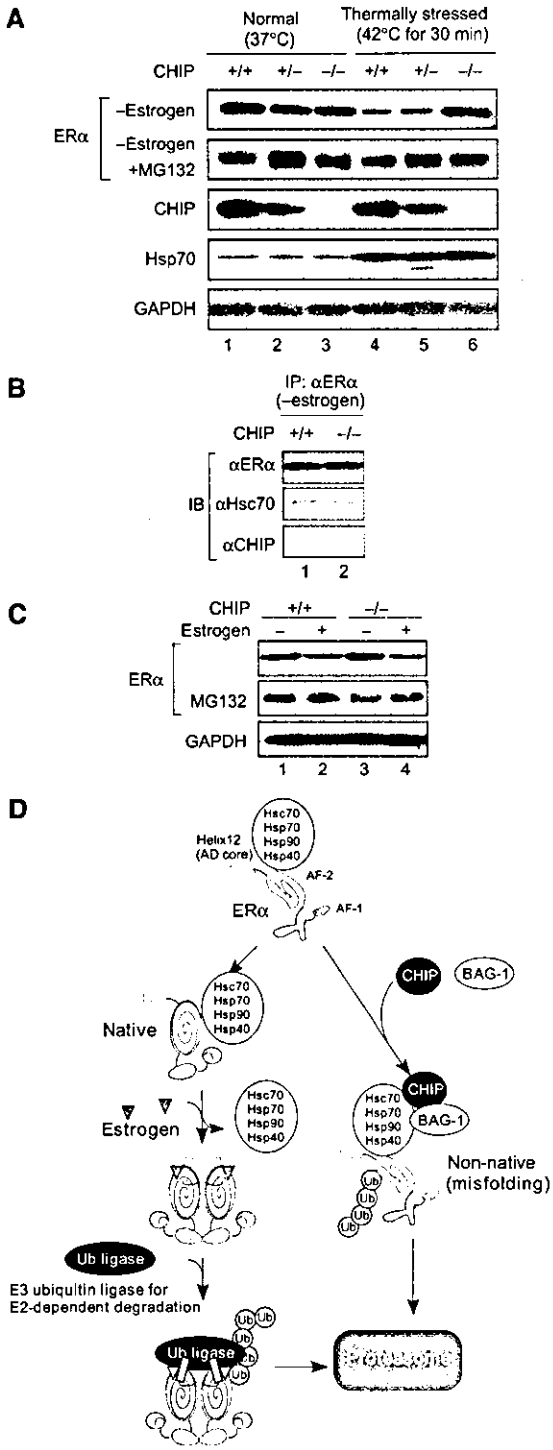

Figure 8 Liganded but not unliganded ERα degradation was observed in CHIP–/– MEF cells. (**A**) Thermally induced degradation of ERα was not observed in CHIP–/– cells. MEF cells were isolated from CHIP–/–, CHIP+/– mice and wild-type littermates (CHIP+/+). MEF cells were cultured under normal conditions (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-ERα antibody in the absence of estrogen. Precipitates were Western blotted with antibodies for ERα, Hsc70 and CHIP. (**C**) Estrogen induced degradation of ERα 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-ERα antibody. (**D**) ERα degradation may be regulated by two independent ubiquitin–proteasome pathways.

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 ERα is subject to proteasome-mediated turnover, which is mechanistically different from the turnover of liganded ERα.

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

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 ERα, we purified proteins using GST-fused ERαLBD, and identified CHIP, which specifically bound to unliganded ERαLBD. Our findings indicate that CHIP binds unliganded ERα 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 ERα 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 ERα, reduced the CHIP-mediated degradation of ERα. These findings confirmed the idea that unliganded ERα 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 ERα 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 ERα 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 ERα 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 ERα requires proteasomal activity (Reid *et al*, 2003). Together with these reports and our observations, it is possible that the binding of estrogen to ERα 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 ERα 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 ERα, 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 ERα preferentially recruited CHIP to ubiquitinate and degrade these receptors under nonpermissive temperatures. In addition, the ubiquitination and degradation of unliganded ERα 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 ERα degradation. These observations provide direct *in vivo* evidence that CHIP selectively ubiquitinated thermally denatured ERα. 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 ERα 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 ERα. 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 μM phenylmethylsulfonyl fluoride (PMSF), 1 μg/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 polyvinylidine 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 ERα 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-ERαLBD 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 ERα 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 ERα and CHIP, and 48 h post-transfection, the cells were labeled for 30 min at 37°C with 50 μCi [^{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-ERα antibody. The immunoprecipitates were resolved by SDS–PAGE and visualized by autoradiography. Phosphoimager was used to quantify the metabolically labeled ERα 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 ERα 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 (Vecter 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 μg/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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Accelerated Publication

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Wnt/β-Catenin and Estrogen Signaling Converge *in Vivo**

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Wnt and estrogen signaling represent important regulatory pathways, each controlling a wide range of biological processes. While an increasing number of observations suggest potential convergence between these pathways, no direct evidence of their functional interaction has been reported. Using human colon and breast cancer cells, we found that estrogen receptor (ER) α- and β-catenin precipitated within the same immunocomplexes, reciprocally enhanced the transactivation of cognate reporter genes, and were reciprocally recruited to cognate response elements in the promoters of endogenous target genes. Using transgenic *Drosophila* that ectopically expressed human ERα alone or together with metabolically stable β-catenin/Armadillo mutants, we demonstrated genetic interaction between these signal transducers *in vivo*. Thus, we present here the first direct evidence of cross-talk between Wnt and estrogen signaling pathways via functional interaction between β-catenin and ERα.

Estrogens regulate a plethora of physiological functions in the developing and adult organism and act predominantly via the activation of ERα[1] and ERβ. Liganded ER dimers bind to promoter estrogen response elements (EREs) and regulate the transcription of target genes. This ER-mediated regulation requires the recruitment of different co-factor complexes and is associated with rearrangement of chromatin structure at EREs within target gene promoters (1, 2). ER can also act as a co-factor at non-ERE sites via interaction with other DNA-bound transcriptional factor complexes, such as c-Jun/c-Fos on the AP-1 site (3) or c-Jun/NFκB on the tumor necrosis factor response element (4). The physiological significance of ERs is demonstrated by the severe abnormalities in development and function of major organs and tissues in mice with ablated ERα and/or ERβ (5). Also, both positive and negative impacts of estrogens in different types of cancer have been well documented (6).

Wnt signaling plays a critical role in numerous processes of development and in adult tissues and appears to be conserved across all animal taxa. β-Catenin is an intracellular transducer of canonical Wnt or Wnt/β-catenin signaling and, thus, has a dual function: as a transcriptional factor and, in a cadherin-bound form, as a regulator of cell adhesion and migration. Cytoplasmic or signaling β-catenin is unstable and rapidly targeted to phosphorylation-ubiquitination-coupled proteasomal degradation. Wnt signaling inhibits this degradation, resulting in the accumulation of β-catenin in the nucleus and its association with members of the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcriptional factors that leads to the activation of Wnt target genes. Mutations that increase the stability of cytoplasmic β-catenin have been implicated in numerous malignant transformations and represent a leading cause of colorectal tumorigenesis (7–9).

Consistent with the concept of morphogen gradients (10) β-catenin exerts different biological effects, such as induction of cell proliferation and apoptosis or stimulation and repression of the same target genes, in a threshold-dependent manner (11, 12). Thus, slight modulation of β-catenin signaling through cross-talk with other pathways may trigger serious physiological consequences. Potential cross-talk between Wnt/β-catenin and estrogen signaling *in vivo* has been implicated in physiological studies on tissues as different as brain (13) and uterus (14). Furthermore, although males and females develop colorectal cancer with approximately the same frequency, its incidence rate is significantly lower in women undergoing hormone replacement therapy (15, 16). While these and other observations suggested the possibility of functional interaction between ER and β-catenin, previous attempts failed to detect such an interaction (13, 17, 18), and no direct evidence of Wnt and estrogen signaling pathway convergence has been reported.

Compared with vertebrates, Wnt signaling has been far better characterized in *Drosophila*, in which it is not obscured by involvement of other, evolutionary more recent multiple pathways. Thus, *Drosophila* provides a powerful experimental system for analysis of functional interaction *in vivo* between Wnt signaling and other regulatory pathways, including those immerged at the later stages of evolution. Therefore, in addition to mammalian cells, to detect functional interaction between Wnt/β-catenin and estrogen signaling *in vivo* we used transgenic *Drosophila* that ectopically expressed human ERα coupled to an ERE-dependent green fluorescent protein (GFP) reporter gene alone or together with constitutively active mutants of Armadillo, a *Drosophila* homologue of β-catenin. Using different approaches, we obtained in this study the first evidence of physical association and transcriptional and genetic interaction *in vivo* between ERα and β-catenin.

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[1] The abbreviations used are: ER, estrogen receptor; ERE, estrogen response element; TCF, T cell factor; LEF, lymphoid enhancer factor; TBE, TCF/LEF binding element; CSFCS, charcoal-stripped fetal calf serum; ChIP, chromatin immunoprecipitation; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; LBD, ligand binding domain; wt, wild-type.

EXPERIMENTAL PROCEDURES

Immunoprecipitation and Immunoblotting—Cells grown in the presence of charcoal-stripped fetal calf serum (CSFCS) were transfected with FLAG-hERα expression vector and harvested 28–30 h post-transfection, after treatment for 3 h with vehicle (ethanol) or 10^{-8} M 17β-estradiol (Sigma), tamoxifen (Sigma), or ICI 182,780 (Tocris). Anti-β-catenin E-5 or H-102 antibodies (Santa Cruz Biotechnology) or preimmune rabbit serum IgG (as a negative control) were used for immunoprecipitation. Western blots were visualized with anti-FLAG M2 (Sigma) or anti-ERα HC-20 (Santa Cruz Biotechnology) antibodies.

Transfection and Reporter Assay—Cells grown in Opti-MEM, 5% CSFCS were transfected with 250 ng of reporter (ERE-tk-luc or tk-luc for MCF7 cells and TOPFLASH or FOPFLASH for colon cancer cells) and 1 ng of pRl (Promega) plasmid (control for transfection efficiency) together with 100 ng of empty (control) or cDNA (β-catenin S33Y for MCF7 cells and ERα for colon cancer cells) expression vector and treated for 16–20 h with vehicle or 10^{-8} M ligand, as indicated. To nullify nonspecific effects on basal promoters, TOPFLASH and ERE-tk-luc reporter activities were normalized against FOPFLASH and tk-Luc reporter activities, respectively, from parallel experiments.

Chromatin Immunoprecipitation (ChIP) Assay—Association of ERα and β-catenin with ERE in the pS2 gene promoter (19) and TCF/LEF binding element (TBE) in the Axin2 gene promoter (20) was analyzed using the Chromatin Immunoprecipitation Kit (Upstate Biotechnology) and HC-20 or E-5 antibody, respectively. As a control for nonspecific chromatin precipitation with these antibodies, a set of primers was used to amplify a pS2 gene DNA segment that does not have ERE or TBE sequences. In addition, IgG from normal preimmune rabbit serum was used as a negative control.

Histology and Immunostaining—All techniques were performed as described previously (21, 22). Expression of ERα and GFP in *Drosophila* eye discs were detected using Zeiss Confocal Laser Scanning System 510 and quantified by calculation of pixels of the corresponding signals using Adobe Photoshop 7 software facilities. TUNEL labeling was performed using the TACS2 TdT-Fluor *In Situ* Apoptosis Detection Kit (Trevigen).

Drosophila Lines and Stocks—The *UAS-ΔArm* and *UAS-ArmS10* mutants were obtained from the Bloomington *Drosophila* Stock Center. Generation and characterization of the used *UAS-ERα, ERE-GFP* transgenic *Drosophila* lines were described in Ref. 23. Briefly, cDNA encoding full-length human ERα, ligand binding domain (LBD) deletion mutant ERα-(1–302), or GFP reporter under control an ERE containing promoter were recloned into the pCaSpeR vector. Transgene constructs together with pπ25.7wc transposase were microinjected into w^{1118} embryos using a micromanipulator (Leica). Several independent transformant lines have been generated. To target ERα expression into the eye disc, transgenic *Drosophila* were crossed with flies of a *GMR-GAL4* line expressing GAL4 driver in the retina under control of the tissue-specific glass multimer gene promoter.

RESULTS

Physical Association of ERα and β-Catenin—Human colon cancer HCT116 cells express metabolically stable β-catenin due to mutation at its putative phosphorylation site. These cells, however, do not express detectable ER. HCT116 cells were transfected with a FLAG-tagged human ERα expression plasmid, and endogenous β-catenin was immunoprecipitated from cell lysates following 3-h preincubation with estrogen or vehicle. IgG from normal rabbit serum was used as a control for nonspecific immunoprecipitation. Obtained immunocomplexes were subjected to Western blotting and analyzed by immunostaining with antibodies against FLAG-tag and ERα.

ERα co-immunoprecipitated with β-catenin even in the absence of ligand; however, ERα-β-catenin association was markedly stimulated by estrogen (Fig. 1*A*). Similar results (data not shown) were obtained using SW480 human colon cancer cells, in which non-mutant β-catenin was stabilized by a loss-of-function mutation in the gene of tumor suppressor Adenomatous polyposis coli, an essential component of the β-catenin degradation machinery. Brief exposure to ligand did not affect FLAG-ERα expression in this (Fig. 1*A*) or further experiments.

As anti-β-catenin antibodies co-precipitated a C-terminally truncated FLAG-ERα-(1–396) (Fig. 1*B*), it appeared that an intact LBD was not essential for the ER interaction with β-catenin. Predictably, C-terminal truncation of ERα abolished the ligand sensitivity of the interaction.

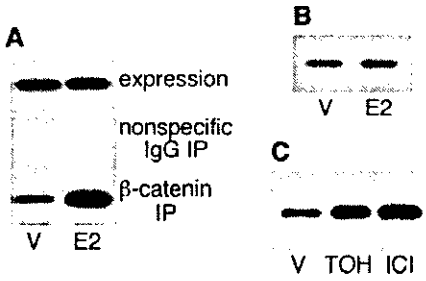

FIG. 1. **Association between ERα and β-catenin in mammalian cells.** *A–C*, anti-FLAG immunostaining of Western blots of immunocomplexes precipitated with antibodies against β-catenin or with nonspecific rabbit IgG (*A*) from HCT116 cells expressing FLAG-tagged full-length ERα (*A, C*) or C-terminally truncated ERα (*B*) pretreated with vehicle (*V*), estradiol (*E2*), tamoxifen (*TOH*), or ICI 182,780 (*ICI*), as indicated. *IP*, immunoprecipitation.

We then analyzed whether ligands that inhibited the transcriptional activity of ERα would also affect its interaction with β-catenin. Immunoprecipitation of ERα with antibodies against β-catenin was significantly stimulated by the ERα partial, tamoxifen, and complete, ICI 182,780, antagonists (Fig. 1*C*).

Transcriptional Interaction between ERα and β-Catenin—Next, we investigated whether the apparent physical association between ERα and β-catenin was consequential for transcriptional function of the proteins. Transactivation of an ERE-dependent reporter by endogenous ER was studied in human breast cancer MCF7 cells, in which the Wnt pathway is practically silent. Expression of stabilized β-catenin S33Y in these cells enhanced ligand-dependent expression of the reporter without affecting its basal activity in the absence of ligand (Fig. 2*A*). Expression of ERα in human colon cancer SW480 (Fig. 2B) and HCT116 (data not shown) cells enhanced the activation of the Wnt-responsive TOPFLASH reporter by endogenous β-catenin in the absence of ligand. Treatment with estrogen resulted in further moderate activation of reporter expression, while ER antagonists appeared not to affect reporter gene activity (Fig. 2*B*).

The reciprocal activation of cognate reporters in the transfection experiments suggested that ERα and β-catenin might reciprocally recruit each other to their corresponding response elements in endogenous target gene promoters. Indeed, antibody against β-catenin precipitated ERE of the pS2 gene promoter from chromatin of β-catenin S33Y expressing MCF7 cells in an estrogen-dependent manner (Fig. 2*C*). Conversely, anti-ERα antibody precipitated in a ligand-dependent manner Axin2 gene promoter putative TBE from chromatin of SW480 cells transfected with an ERα expression construct, while recruitment of β-catenin to the TBE was not sensitive to the presence of estrogen (Fig. 2*D*). The used antibodies did not display nonspecific chromatin precipitation (Fig. 2*E*).

Consistent with the results obtained using MCF7 cells, ERα transactivation was markedly enhanced *in vivo* by the stabilized Armadillo mutants ΔArm (24) (Fig. 2*F*) or ArmS10 (25) (data not shown) when ectopically co-expressed in the *Drosophila* eye disc.

Genetic Interaction between ERα and β-Catenin—Constitutive activation of Armadillo in the *Drosophila* eye disc has been shown to induce apoptosis and consequent degeneration in the adult eye (26, 27). Potentiation of β-catenin transcriptional activity by ERα in SW480 cells (Fig. 2*B*) and functional interaction between ERα and Armadillo (Fig. 2*F*) would predict activation of endogenous Armadillo by the ectopic ERα expression in

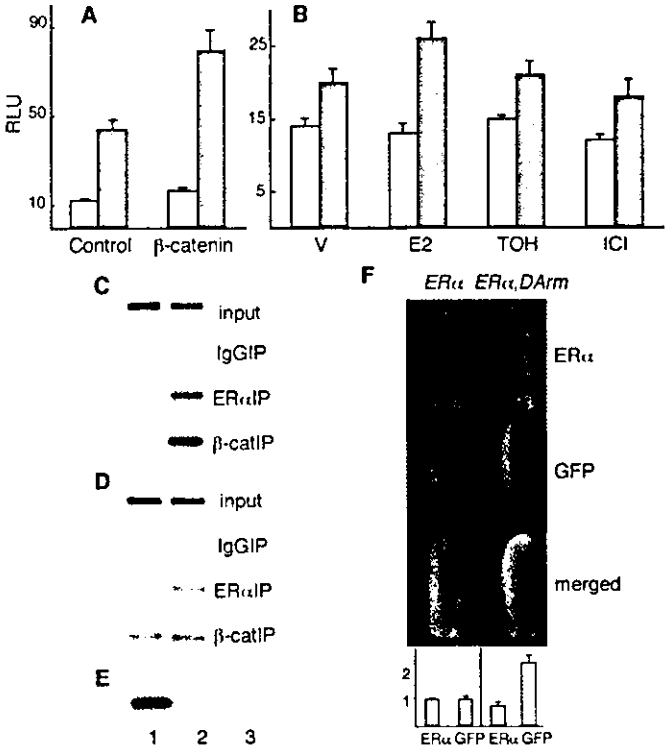

FIG. 2. **Transcriptional interaction between ERα and β-catenin.** *A*, MCF7 cells were transfected with ERE-tk-Luc reporter construct together with empty (*Control*) or β-catenin S33Y expression vector and treated with vehicle or estradiol (*open* and *filled bars*, respectively). *B*, SW480 cells were co-transfected with Wnt/β-catenin-responsive reporter and empty or ERα expression vector (*open* and *filled bars*, respectively) and treated with vehicle (*V*), estradiol (*E2*), tamoxifen (*TOH*), or ICI 182,780 (ICI), as indicated. The data represent the mean ± S.D. of three independent experiments. *C* and *D*, ChIP assay of the putative ERE of the pS2 gene promoter in MCF7 cells (*C*) and the putative TBE of the Axin2 gene promoter in SW480 cells (*D*) with anti-ERα (*ERαIP*) or β-catenin (*β-catIP*) antibodies or preimmune rabbit IgG (*IgGIP*) as a negative control. Cells were pretreated for 3 h with vehicle (*left column*) or estradiol (*right column*). *E*, control for a nonspecific chromatin immunoprecipitation: amplification of a pS2 gene DNA segment that does not contain ERE or TBE sequences from DNA samples used for PCR presented in C, *right column*: input (*lane 1*), ChIP with anti-ERα (*lane 2*), or anti-β-catenin (*lane 3*) antibodies. The data shown are representative of typical results of at least three independent ChIP experiments. *F*, estrogen-induced expression of an ERE-dependent GFP reporter (*green*) in *Drosophila* third instar larva eye discs ectopically expressing human ERα (*red*) alone or together with constitutively active Armadillo mutant ΔArm. Similar results were obtained with a different constitutively active Armadillo mutant, ArmS10.

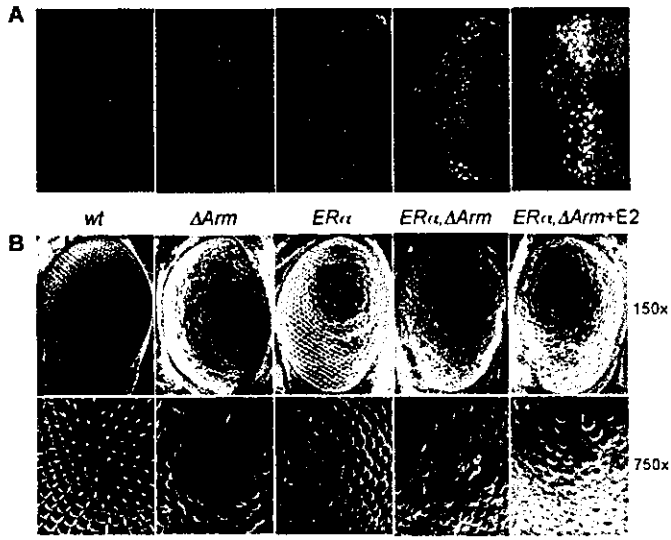

FIG. 3. **Genetic interaction between ERα and β-catenin/Armadillo in *Drosophila*.** *A*, apoptosis (*green*, fluorescein isothiocyanate TUNEL-labeled cells) in third instar larval eye discs; *B*, scanning electron microscope images of adult eye from *Drosophila* with ectopic expression of ERα, stabilized Armadillo mutant ΔArm, or both, as indicated. The same phenotypes were produced in experiments using other independently obtained *Drosophila* lines with different chromosomal localization of the ERα transgene.

the *Drosophila* eye disc leading to development of a phenotype characteristic of abnormal Wnt/β-catenin activation.

We performed TUNEL staining of the third instar larval eye discs with ectopic expression of ERα alone or together with the constitutively active Armadillo mutant ΔArm. When expressed singly, ERα and ΔArm both induced a slight increase in apoptosis compared with wild-type (wt) eye discs from *Drosophila* of the parental line. Co-expression of ERα and ΔArm resulted in a marked increase in apoptotic cell number. Importantly, while estrogen had no discernible effect on apoptosis in wt eye discs and those expressing either ERα or ΔArm alone (data not shown), treatment with estradiol significantly increased apoptosis rates when ERα and ΔArm (*ERα,ΔArm*+E2) were co-expressed (Fig. 3*A*). Activated Armadillo has a mild apoptotic effect in the third instar larva eye disc, reportedly due to the protective counteraction at this stage by the EGFR/MAPK signaling (27). This allowed us to detect differences in apoptosis patterns in transgenic fly eye discs at this developmental stage that would otherwise be difficult to distinguish due to the onset of massive cell death at the later stages.

We compared adult eye phenotypes of flies from these transgenic lines and the wt (Fig. 3*B*). The normal *Drosophila* eye is composed by regularly spaced ommatidia with regularly oriented interommatidial bristles. Expression of ERα in the eye disc leads to development of phenotypes similar to those caused by expression of ΔArm: rough eye appearance and disorientation or loss of interommatidial bristles. Co-expression of ΔArm and ERα synergistically enhanced this abnormal eye development. Again, while estradiol appeared not to affect the separate ΔArm or ERα expression phenotypes (data not shown), treatment with estrogen, however, further aggravated the severity of eye abnormalities in the ERα and ΔArm co-expression mutants (*ERα,ΔArm*+E2). Consistent with β-catenin-ERα-(1–396) co-immunoprecipitation, ectopic expression of the LBD deletion mutant ERα-(1–302) in the eye disc produced a phenotype closely resembling that with the full-length ERα (data not shown).

DISCUSSION

We found that β-catenin associated with ERα even in the absence of ligand and that estrogens further enhanced this interaction. While it is possible that the ligand-independent association was due, at least in part, to the overexpression of one of the interacting proteins, the association between β-catenin and C-terminally truncated ERα suggested that the ligand binding was not essential but might rather induce a more favorable conformation for ERα to interact with β-catenin. This may be of functional significance at physiological concentrations of the interacting proteins. Interestingly, β-catenin recruitment to EREs and ERα recruitment to TBEs in the promoters of endogenous target genes were both highly ligand-dependent. The apparently equal stimulation of ERα-β-catenin interaction by ER agonists and antagonists may have important implications for the design of novel therapeutic strategies.

Our most significant finding was that ERα functionally interacted with β-catenin/Armadillo *in vivo* in transgenic *Drosophila*. The ligand-dependent transactivation function of ERα was significantly enhanced by the co-expression of stabilized Armadillo mutants. Abnormalities in the eye development in-