Human Phosphatidylethanolamine-binding Protein 4 Promotes Transactivation of Estrogen Receptor α (ERα) in Human Cancer Cells by Inhibiting Proteasome-dependent ERα Degradation via Association with Src

Haibo Liu1, Jianming Qiu2, Nan Li3, Taoyong Chen4, and Xuetao Cao5,2

From the 1National Key Laboratory of Medical Immunology and Institute of Immunology, Second Military Medical University, Shanghai 200433 and the 2Institute of Immunology, Zhejiang University School of Medicine, Hangzhou 310058, China

We identified human phosphatidylethanolamine-binding protein 4 (hPEBP4) as a human-derived novel member of the phosphatidylethanolamine-binding protein family, which is involved in apoptosis resistance of tumor cells. Because of its preferential expression in estrogen-related cancers, we wondered whether hPEBP4 plays a role in estrogen-induced cancer cell growth. Here, we demonstrated that hPEBP4 inhibited the 17β-estradiol (E2)-induced, proteasome-dependent estrogen receptor α (ERα) degradation to increase the protein level of ERα. Silencing of hPEBP4 inhibited the recruitment of ERα to the promoter of the ERα target gene pS2 in MCF-7 breast cancer cells after E2 treatment. E2-induced, ERα-mediated transcription via the estrogen-response element, as well as the cellular proliferation, was significantly suppressed in hPEBP4-silenced MCF-7 cells. We found that Src, whose association with ERα facilitates the ERα binding to components of proteolytic machinery, could associate with hPEBP4 and that overexpression of hPEBP4 prevented the E2-induced interaction between ERα and Src. ERα overexpression, proteasome inhibitor, or Src inhibitor could reverse the suppression of ERα-mediated transactivation by hPEBP4 silencing. The inhibition of the proteasome degradation and the promotion of transactivation of ERα by hPEBP4 via the Src pathway were further confirmed in HeLa cells. Finally, we found that the promoting effects of hPEBP4 on ERα-mediated transactivation and estrogen-induced proliferation of cancer cells did not depend on its regulation of Akt and ERK activity. Our data suggest that hPEBP4 inhibits proteasome-dependent ERα degradation through the Src pathway, thus enhancing ERα-mediated transactivation and promoting the proliferation of cancer cells in response to estrogen.

Estrogen is involved in the growth and differentiation of diverse tissues (1). As a potent mitogen, estrogen accounts for

---

*This work was supported by National High Biotechnology Development Program of China Grants 2006AA02A305, 2009ZX09503-003, and 2009AA02Z101, National Key Basic Research Program of China Grant 2010CB911903, National Natural Science Foundation of China Grants 30721091 and 30772504, and Shanghai Committee of Science and Technology Grants 09QH1402800 and 09SG35.

1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

2 To whom correspondence should be addressed. Tel.: 86-21-5562-0605; Fax: 86-21-6538-2502; E-mail: caoxt@immunol.org.

3 The abbreviations used are: ERα, estrogen receptor α; E2, 17β-estradiol; h, human; BrdUrd, bromodeoxyuridine; ERK, extracellular signal-regulated kinase; DMEM, Dulbecco’s modified Eagle’s medium; DCC-FCS, FCS treated with dextran-coated charcoal; siRNA, small interfering RNA; TNF-α, tumor necrosis factor; Akt, proto-oncogene serine/threonine-protein kinase; pS2, estrogen-responsive gene; pS2, estrogen-responsive gene; MCF-7, human breast adenocarcinoma cell line; HeLa, human cervical carcinoma cell line; Src, src proto-oncogene

© 2010 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
tumors. Therefore, the exact role of hPEBP4 in carcinogenesis still remains unknown. Interestingly, we find that all types of tumors, including breast and ovarian tumors, in which hPEBP4 is endogenously overexpressed, have been believed to associate with estrogen during their development. Therefore, we speculate that hPEBP4 may be involved in the responsiveness of cancer cells to estrogen in the tumor microenvironment.

Cellular Src (c-Src), the 60-kDa tyrosine kinase, regulates cellular proliferation and tumor metastasis (14). Recent studies have shown that Src promotes cell proliferation by driving ERα transcripational activity and targeting ERα for ubiquitin-dependent proteolysis in human breast cancers (15). The cross-talk between Src and liganded ERα may affect the therapeutic efficacy and prognosis in patients with breast cancer.

In this study, we focused on the role of hPEBP4 in estrogen-induced ERα degradation in human cancer cells. To do this, RNA interference was used to reduce hPEBP4 expression in MCF-7 breast cancer cells, and the effects of hPEBP4 on ERα degradation and transcriptional activity were investigated. Our results indicated that hPEBP4 inhibits proteasome-dependent degradation of ERα via the Src pathway and thus promotes ERα-mediated transactivation. This phenomenon is also further confirmed in HeLa cells that are forced to express hPEBP4. The inhibitory effects of hPEBP4 may be attributed to its association with Src, which may disrupt the interaction between Src and ERα. We also found that the promoting effects of hPEBP4 on ERα-mediated transactivation and estrogen-induced proliferation of cancer cells are independent of its roles in regulating ERK or Akt kinase activity. These results suggest that hPEBP4 may represent a novel regulatory mechanism of the ERα signal pathway in cancer cells, which offers a new therapeutic target for hormone-responsive cancers.

EXPERIMENTAL PROCEDURES

Reagents and Plasmids—17β-Estradiol (E2), MG-132, and CHX were purchased from Sigma. MEK1 inhibitor U0126 and PI3K inhibitor LY294002 were obtained from New England Biolabs (Beverly, MA) and Calbiochem, respectively. Src inhibitor PP1 was from Santa Cruz Biotechnology. Antibodies specific for ERα (MC-20), phospho-ERα (Tyr-537), AIB-1, SRC-1, GRIP1, MDM2, and cyclin D1 were from Santa Cruz Biotechnology. Antibodies specific for phospho-Src (Tyr-416), phospho-ERK1/2, phospho-Akt, Src, and horseradish peroxidase-conjugated secondary antibodies were from Cell Signaling Technology. Vector for full-length and truncated hPEBP4, hPEBP4-B, and p75-B (amino acids 1–75 of hPEBP4, lacking the highly conserved phosphatidyethanolamine-binding domain) were constructed as described previously (10). Human pSG5-ERα (16) and the 3xERE-TATA-Luc reporter constructs (17) were kindly provided by Dr. Ingemar Pongratz (Karolinska Institute). Human wild-type MDM2 (MDM2-WT) and domain-negative mutant MDM2.S166A/S186A (MDM2-DN) plasmids were kindly provided by Dr. Alex Toker (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston). Plasmid encoding activated Src (Src Y530F) was constructed by PCR amplification. siRNA-resistant hPEBP4 expression vector (hPEBP4-R) was generated by PCR-directed mutagenesis with a 2-bp mutation in the siRNA-targeted regions to fully abolish the siRNA effect. All constructs were confirmed by DNA sequencing and/or Western blot analysis using appropriate antibodies.

Cell Culture—Human breast cancer cell line MCF-7 and human cervix cancer cell line HeLa were obtained from American Type Culture Collection (ATCC) and grown in phenol red-free DMEM supplemented with 5% DCC-FCS (FCS treated with dextran-coated charcoal) (Hyclone), 4.5 g/liter D-glucose, nonessential amino acids (100 µmol/liter each), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mmol/liter glutamine at 37 °C in a 5% CO₂ atmosphere.

RNA Interference of hPEBP4—hPEBP4 transient RNA interference with chemically synthesized siRNA duplex (GeneChem, Shanghai, China) and mutation control was performed using Lipofectamine 2000 reagent (Invitrogen) as described previously (10). Efficacy of interference was determined by Western blot 48 h after transient transfection of siRNAs.

Assay for Luciferase Reporter Activity—Cells were seeded at 5 × 10⁴ cells/well in 24-well plates 16 h prior to transfection. Each well was transfected with 3xERE-TATA-Luc plasmid and pGL-TK control construct and treated as indicated. Luciferase activity was measured using the Dual-Luciferase reporter assay kit (Promega). Data were normalized for transfection efficiency by dividing firefly luciferase activity with that of Renilla luciferase (18, 19).

Real Time PCR—Cells were grown for 48 h in phenol red-free DMEM supplemented with 5% DCC-FCS serum after RNA interference and treated with E₂ at a final concentration of 10 nm. RNA was collected with TRizol (Invitrogen) as described previously (20, 21). cDNA was synthesized with 1 µg of total RNA using first strand cDNA synthesis kit ReverTra Ace-α (Toyobo) at 42 and 99 °C for 20 and 5 min, respectively. Quantification of inducible expression of pS2 was performed using SYBR master mix (Toyobo) on an iCycler fitted with an optical assembly unit (Bio-Rad). The relative expression of pS2 mRNA was calculated using the 2⁻ΔΔCt method with β-actin mRNA as an internal control (22). The primers used were as follows: 5’-ATACATCGAGCTCCCTCTCA-3’ (forward) and 5’-AAGCGTGTCAGAACTTCC-3’ (reverse) for pS2; 5’-TTCGACGTACAAAGAGAG-3’ (forward) and 5’-GGCTGTAGCTTCTTCCA-3’ (reverse) for β-actin.

Chromatin Immunoprecipitation—MCF-7 cells were subjected to RNA interference and grown for 48 h in phenol red-free DMEM supplemented with 5% DCC-FCS. Cells were then treated with E₂ at 10 nm for the indicated time and then fixed with formaldehyde. Cells were lysed and sonicated, and 10 µl of purified chromatin samples was used as inputs, and the remaining amount was subjected to the chromatin immunoprecipitation procedure. Purified chromatin samples were immunoprecipitated with 1 µg of anti-ERα antibody or antibodies against AIB-1, SRC-1, GRIP1. The immune complexes were recovered using protein A/G-Sepharose (Calbiochem) and processed as described (23). The immunoprecipitated DNA was amplified by PCR with the pS2 promoter-specific primer, and the pS2 upstream primer pair was used as negative control (24).

Western Blot and CHX Chase—A BCA protein assay reagent kit (Pierce) was used to measure protein concentration. Sam-
hPEBP4 Inhibits ERα Degradation via Src

**FIGURE 1. Silencing of hPEBP4 increases ERα degradation to repress ERα expression.** A and B, MCF-7 cells were transfected with hPEBP4-specific siRNA (hPEBP4 siRNA) or mutated hPEBP4 siRNA (mutation control) and cultured in the absence of E₂ for 48 h. Then the cells were treated without or with E₂ (10 nM) for the indicated time and subjected to Western blot analysis using indicated antibodies. C, hPEBP4-silenced MCF-7 cells were cultured in the absence of E₂ for 48 h and treated with MG-132 (10 μM) or DMSO 1 h prior to 10 nM E₂ stimulation for 4 h. Western blot was then performed with anti-ERα antibody. D, hPEBP4-silenced MCF-7 cells were cultured in the absence of E₂ for 48 h. ERα was assayed by CHX chase at 2, 4, and 6 h after treatment with or without E₂. E, hPEBP4-silenced MCF-7 cells were transfected with siRNA-resistant hPEBP4 expression vector (hPEBP4-R) or mock vector, cultured for 24 h, and then treated with E₂ (10 nM) for 6 h. Western blot was performed with total cell lysates using indicated antibodies.

RESULTS

Silencing of hPEBP4 Attenuates the Expression of ERα by Increasing ERα Degradation—We first determined the effect of hPEBP4 down-regulation on ERα levels in MCF-7 breast cancer cells upon E₂ stimulation. The result confirmed that siRNA could efficiently reduce hPEBP4 protein levels in MCF-7 cells (Fig. 1A). The cells were then grown in phenol red-free DMEM with 5% DCC-FCS for 2 days before treating with E₂ or ethyl alcohol as a vehicle control. As shown in Fig. 1B, E₂ stimulation elicited an up-regulation of ERα protein level, which is in agreement that reported previously (9). Targeted reduction of hPEBP4 with siRNA dramatically inhibited the increase of ERα expression as compared with siRNA mutation control (Fig. 1B). Additionally, hPEBP4 down-regulation-mediated depletion of ERα was associated with attenuation of the progrowth cyclin D1 protein (Fig. 1B), a downstream target of ERα. The changes of ERα protein level were independent of mRNA, which did not show any concomitant change within the duration of E₂ stimulation (supplemental Fig. S1), suggesting that hPEBP4 may affect the ERα protein expression at the post-transcriptional level. As it has been determined that proteasome-mediated degradation acts as an important mechanism in fine-tuning the ERα-initiated signal pathway (25), we used MG-132, a specific proteasome inhibitor, to test whether the effect of hPEBP4 on ERα protein level was caused by affecting proteasome-dependent proteolysis activity. The results showed that inhibiting proteasome activity with MG-132 led to an accumulation of the ERα protein and that MG-132 treatment almost completely abolished the reduction of ERα caused by hPEBP4 silence (Fig. 1C). In support of this, we examined the effect of hPEBP4 on ERα stability by the CHX chase assay. As shown in Fig. 1D, a decrease in ERα stability was found in hPEBP4-silenced cells when compared with that of mutation control cells (Fig. 1D, top). A more significant effect of hPEBP4 on ERα stability was observed in the presence of E₂ (Fig. 1D, bottom).

We next performed rescue experiments to further confirm the effect of hPEBP4 silencing on ERα degradation. The siRNA-resistant hPEBP4 expression vector (hPEBP4-R) was constructed and transfected into hPEBP4 siRNA-transfected samples containing equal amounts of protein were prepared, separated by 12% SDS-PAGE, and transferred to Protran nitrocellulose membranes. The blots were probed with specific antibodies as indicated with appropriate horseradish peroxidase-conjugated antibodies as secondary antibodies. Supersignal West Femto maximum sensitivity substrate (Pierce) was used for the chemiluminescent visualization of membrane-bound proteins. The ERα t1/2 was determined by CHX chase, with addition of 100 μg of CHX considered t = 0. Cells were lysed at the times as indicated, and equal samples were analyzed by immunoblotting.

Detection of ERα Ubiquitylation—MCF-7 cells were starved in 0.1% DCC-FCS for 48 h and then treated 5% DCC-FCS plus 10 nM E₂ for 6 h with or without prior addition of Src inhibitor PP1. Total cell lysates were collected, and ERα was determined by Western blot. To analyze ubiquitylated ERα, ERα was immunoprecipitated, and precipitates were blotted with antibody against ubiquitin (Abcam). Coimmunoprecipitation—Cellular lysates of stably transfected HeLa cells were precleared with protein A-Sepharose beads (Calbiochem), and immunoprecipitation was performed as described previously (11, 20) using ERα (Santa Cruz Biotechnology), Src, and Myc (Cell Signaling Technology) antibodies cross-linked to protein-A-Sepharose beads. Resulting elution was subjected to Western blot to detect coimmunoprecipitated proteins.

BrdUrd Incorporation Assay—The protocol was performed as described previously (18, 19). Briefly, MCF-7 cells were seeded in 6-well plate, stimulated with 10 nM E₂ and incubated with 10 μM BrdUrd (Calbiochem) for 2 h before collection. The cells were then fixed, permeabilized, incubated with fluorescein isothiocyanate-conjugated antibody, and subjected to fluorescence-activated cell sorting analysis.

Statistical Analysis—The Student’s t test was used to determine the statistical significance of the data obtained and to compare the means between groups. A p value of less than 0.05 represented a statistically significant difference.
hPEBP4 Inhibits ERα Degradation via Src

Treatment with 10 nM E2 showed a marked increase in luciferase transcription and activity, which was significantly suppressed by hPEBP4 silencing (Fig. 2A). The E2-induced expression of pS2, the endogenous target gene transcriptionally activated by ERα, was also determined, and the results showed that the pS2 mRNA level was down-regulated in the hPEBP4-silenced MCF-7 cells (Fig. 2B). The amount of ERα as well as its cofactors recruited to estrogen-responsive promoter of pS2 was also examined by chromatin immunoprecipitation. As shown in Fig. 2C, we found that E2 stimulation resulted in a dramatic increase of ERα binding with the estrogen-responsive promoter, and silencing of hPEBP4 significantly reduced the amount of ERα as well as its cofactors (SRC-1, AIB-1, and GRIP1) bound to the estrogen-responsive promoter of pS2 after E2 treatment. We went further to evaluate the effect of hPEBP4 on E2-induced cancer cell proliferation using BrdUrd incorporation assay. We found that the percentage of proliferative MCF-7 cells was markedly reduced after hPEBP4 was silenced (Fig. 2D). In addition, to determine whether it is ERα degradation that causes the difference in cancer cell proliferation, we transfected MCF-7 cells with ERα plasmid. We found that forced expression of ERα completely reversed the decreased proliferation of hPEBP4-silenced MCF-7 cells, suggesting that hPEBP4 promotes E2-induced proliferation of cancer cells as a consequence of inhibiting ERα degradation (Fig. 2D). Therefore, depletion of hPEBP4 suppressed both ERα-mediated transcriptional activity and cellular proliferation induced by E2.

To confirm that hPEBP4 does promote ERα-mediated transactivation by inhibiting proteasome, we determined the effect of MG-132 on ERα-mediated transactivation using reporter gene assay. The result showed that inhibiting proteasome activity with MG-132 reversed the decrease in transcriptional activity of hPEBP4-silenced cells (Fig. 2E). When MCF-7 cells were transiently transfected with pSG5-ERα plasmid after silencing hPEBP4, the E2-induced increase in luciferase activity and BrdUrd incorporation was reversed by proteasome inhibitor MG-132 (Fig. 2F). The data indicated that hPEBP4-mediated inhibition of ERα transactivation is through inhibition of ERα degradation via Src-dependent proteasomal degradation.
hPEBP4 Inhibits ERα Degradation via Src

2F). Together, these data suggest that hPEBP4-mediated inhibition of proteasome-dependent ERα degradation potentiates ERα-mediated transactivation.

hPEBP4 Overexpression Up-regulates ERα-mediated Transactivation and Cellular Proliferation Induced by E2 in HeLa Cells—We further confirmed the above effects of hPEBP4 by using HeLa cells as a cell model. Because both hPEBP4 and ERα are not endogenously expressed in HeLa cells, we first established HeLa cells stably expressing hPEBP4 and then transfected with pSG5-ERα plasmid for 48 h to transiently express ERα. Western blot was used to verify the forced expression of hPEBP4 and transient expression of ERα in HeLa cells (Fig. 3A). As shown in Fig. 3, B and C, overexpression of hPEBP4 led to increased levels of ERα protein, as well as the ERα-mediated transcriptional activity. hPEBP4 overexpression also resulted in a higher proportion of proliferative HeLa cells (Fig. 3D). Once proteasome activity was blocked with MG-132, no promoting effect of hPEBP4 was observed on the ERα protein level as well as E2-induced transactivation (Fig. 3, C and E). Thus, consistent with the results of hPEBP4 silencing in MCF-7 cells, our findings indicated that overexpression of hPEBP4 in HeLa cells promoted ERα-mediated transactivation and cellular proliferation induced by E2 via inhibiting proteasome-dependent ERα degradation.

hPEBP4 Suppresses Src Activation and Disrupts the Interaction between Src and ERα via Association with Src—It has been reported that Src could promote ERα transcriptional activity and target ERα for ubiquitin-mediated proteolysis (15). We next tested the effect of hPEBP4 on Src activation. As shown in Fig. 4A, E2 induced Src phosphorylation in a time-dependent manner. hPEBP4-silenced cells showed a more evident basal Src phosphorylation compared with that of mutation of control cells. The extent of Src phosphorylation was greater in hPEBP4-silenced cells compared with mutation of control cells during the entire time course, suggesting that silencing of hPEBP4 increased the activation of Src. Besides, the phosphorylation of ERα on Tyr-537, which is dependent on Src kinase and may then enhance E2 binding to ER (26, 27), was also increased after hPEBP4 silencing (Fig. 4A). We then evaluated the role of increased Src activity in the hPEBP4-mediated regulation of ERα protein expression. We found that treatment of MCF-7 cells with the Src inhibitor PP1 caused a dose-dependent increase of ERα protein levels over 36 h (supplemental Fig. S2). PP1 could restore the reduction of ERα and cyclin D1 induced by hPEBP4 depletion after E2 stimulation in MCF-7 cells (Fig. 4B), indicating the involvement of Src in the regulation of ERα protein expression and its transcriptional activity by hPEBP4. As indicated in Fig. 4C, hPEBP4-silenced cells showed a more profound ubiquitination level of ERα after E2 stimulation compared with mutation of control cells, which could be inhibited by PP1. Accordingly, the ERα protein level was inversely correlated with its ubiquitination level. More importantly, PP1 pretreatment could fully restore the decrease of ERα transcriptional activity induced by hPEBP4 silencing (Fig. 4D). These results strongly suggested that the decrease in ERα level induced by hPEBP4 silence may be attributed to the increased ubiquitination level because of the up-regulated Src activity. Unexpectedly, Src inhibition repressed ERα transcriptional activity in mutation control cells, suggesting that moderate activity of Src was required for efficient ERα transactivation. In HeLa cells, we found that overexpression of hPEBP4 significantly inhibited the activation of Src after E2 stimulation (Fig. 4E). Forced expression of activated Src blocked the up-regulation of ERα induced by hPEBP4 overexpression (Fig. 4F). Therefore, the promoting effect of hPEBP4
hPEBP4 Inhibits ERα Degradation via Src

FIGURE 4. Src inhibitor PP1 reverses the inhibition of ERα protein level and ERα-mediated transactivation by hPEBP4 knockdown. A and B, hPEBP4-silenced MCF-7 cells were cultured in the absence of E2 for 48 h. The cells were then pretreated with or without Src inhibitor PP1 (10 μM) before the stimulation of E2 for the indicated times. Western blot was performed with total cell lysates using the indicated antibodies. C, MCF-7 cells were cultured in 0.1% DCC-FCS and estrogen-deprived conditions for 48 h to deplete both growth factors and estrogen, and then cells were treated with PP1 for 10 min prior to a 6-h stimulation with E2 and 5% DCC-FCS. ERα and phospho-Src levels were determined by Western blot. For measurement of ERα ubiquitination, ERα was precipitated and blotted with anti-ubiquitin antibody. D, hPEBP4-silenced MCF-7 cells were transfected with luciferase reporter plasmids of ERE and pGL-TK vector, cultured for 7 h, and then treated with or without PP1 for 1 h prior to stimulation with E2 (10 nM) for 16 h. The luciferase activity was measured using the Dual-Luciferase reporter assay system. E and F, stably transfected HeLa cells were transiently transfected with pSG5-ERα together with or without the activated Src plasmid (Src Y530F). The cells were cultured in the absence of E2 for 48 h and then stimulated with E2 (10 nM) for the indicated time (C) or for 4 h (F). Western blots were performed with total cell lysates by using the indicated antibodies. Data show means ± S.D. of triplicates from one experiment representative of three experiments. *p < 0.05.

on ERα-mediated transactivation via Src pathway is not restricted to MCF-7 breast cancer cells.

To further determine the correlation between hPEBP4 and Src, we performed coimmunoprecipitation to examine the association of the two molecules. Stably transfected HeLa cells were stimulated with E2 and immunoprecipitated with either ERα or Src antibody. As shown in Fig. 5A, the association of ERα and Src was detected in both control and hPEBP4-overexpressing cells after E2 stimulation. However, overexpression of hPEBP4 apparently reduced the interaction between ERα and Src. hPEBP4 associated with Src, but it failed to associate with ERα (Fig. 5A). We also found that p75, the construct lacking the phosphatidyethanolamine-binding domain of hPEBP5, could not immunoprecipitated with Src (Fig. 5B). These results suggest that hPEBP4 may disrupt the interaction between ERα and Src via association with Src.

hPEBP4 Promotes ERα-mediated Transactivation and E2-induced Proliferation in a ERK- and Akt-independent Pathway—It is known that ERα activity can be regulated in a non-genomic pathway via protein-protein interactions. Several studies have shown that ERα can activate Src and its downstream kinases ERK and Akt (28, 29). These pathways are critical in the proliferation and survival of breast cancer cells. Because hPEBP4 was found to be associated with Src, we then tested the effect of hPEBP4 on the activation of two major components—ERK and Akt in estrogen-mediated nongenomic signal pathway. Results of Western blot showed that E2 could activate ERK but only slightly activate Akt in MCF-7 cells. Silencing of hPEBP4 significantly enhanced ERK activation but reduced Akt activation after treatment with E2 (Fig. 6A).

We then examined the ERα-mediated transactivation after blocking the activity of ERK and Akt with U0126 and LY294002, respectively. As shown in Fig. 6B, we found that inhibiting ERK caused a reduction in ERE-luciferase activity (p < 0.05) but inhibiting Akt yielded no obvious effect. However, neither U0126 nor LY294002 could block the suppression effect of hPEBP4 silencing on ERα-mediated transactivation. We also examined the accompanying ERα protein level after treatment with inhibitors for ERK and Akt. We found U0126 and LY294002 could not rescue the drop of ERα level caused by hPEBP4 silencing (Fig. 6C). These results imply that hPEBP4 also participates in the regulation of Akt and ERK in ERα-mediated nongenomic signal pathway, but the promoting effect of hPEBP4 on the ERα-mediated transactivation does not depend on its regulation of ERK and Akt activity.
DISCUSSION
hPEBP4 was first identified by us as an anti-apoptotic molecule and a potential target for treatment of breast cancer (10–13). By silencing hPEBP4 in MCF-7 cells and overexpressing hPEBP4 in HeLa cells, we have demonstrated that hPEBP4 suppresses the Src activation and disrupts the interaction between Src and ERα via association with Src, resulting in inhibition of proteasome-dependent degradation of ERα and promotion of ERα-mediated transactivation of a targeted gene such as pS2 and cyclin D1. These molecular perturbations may be responsible for the promoting effect of hPEBP4 on E2-induced cellular proliferation mediated by ERα. Degradation of ERα may be part of the physiological negative feedback during the interaction of estrogen and estrogen-responsive tissues. However, cancer cells with genetic instability will probably evolve various means to minimize the “side effect” of residual feedback in malignant development. Up-regulation of hPEBP4 in several estrogen-related tumors, as demonstrated previously by us (10, 12), may be one of the means for ER-related tumors to avoid ERα degradation. By inhibiting the degradation of ERα, hPEBP4 sustains the ERα at a high level and facilitates the carcinogenic effect of estrogen.

Degradation of ERα is an important mechanism in the regulation of ERα activity. Previous studies have suggested that ERα phosphorylation by several kinases can promote its degradation. It has been reported that E2-induced rapid activation of ERK and Akt can promote ERα-mediated transactivation by phosphorylating coactivators of ERα or ERα itself (30, 31). However, our study showed that neither ERK nor Akt accounts for the promoting effect of hPEBP4 on ERα-mediated transactivation because inhibiting ERK or Akt could not rescue the increased degradation of ERα caused by hPEBP4 silence, which is consistent with our previous study showing that blockage of PI3K or ERK does not alter the ERα level when bound to E2 (32). Instead, our data suggest that Src may be responsible for hPEBP4-induced elevation of ERα levels. ERα phosphorylation by Src kinase can increase its affinity for estrogen (26) and therefore enhance the ERα-coactivator binding and transcriptional activity of ERα. Also, Src appears to promote ERα ubiquitylation because Src inhibition impaired cellular ERα ubiquitylation and proteolysis in vivo (33). Therefore, liganded ERα may recruit Src or Src-dependent kinases, leading to phosphorylation of ERα that facilitates ERα binding to coactivators and/or components of the proteolytic machinery (15). Our results suggested that hPEBP4 could inhibit the interaction between Src and ERα, thereby inhibiting the Src-dependent degradation of ERα. However, we are not sure whether the reduced interaction between Src and ERα can be attributed to down-regulated activity of...
Src itself induced by hPEBP4 overexpression. Glutathione S-transferase pulldown assay showed that GST-hPEBP4 fusion protein could not pull down either Src or ERα (data not shown), indicating the interaction between Src and hPEBP4 may be indirect or dynamic. Besides, Src or its downstream effectors may also regulate ligand-activated ERα coactivator phosphorylation to affect ERα degradation, because several ERα coactivators are known to be ubiquitin ligases (34, 35). We have found that the E2-induced phosphorylation of MDM2, an E3 ligase and also a direct activator of ERα (35), was inhibited by hPEBP4 overexpression and increased by hPEBP4 silencing (supplemental Fig S3, A and B). Wild-type MDM2 overexpression could completely abolish the effect of hPEBP4 on ERα-mediated transactivation in HeLa cells, whereas mutant MDM2 S166A/S186A could not (supplemental Fig S3C), indicating the critical role of the E3 ligase MDM2 in the regulation of ERα-mediated transactivation by hPEBP4. Our data support the notion that hPEBP4 regulates ERα activity via association with Src in estrogens-mediated nongenomic pathway. Nevertheless, how hPEBP4 affects Src-dependent ERα degradation remains to be further investigated.

The results showed here are in contrast to other reports that show an inverse relationship between receptor proteolysis and transcriptional efficiency (36, 37) or a dissociation of ERα activation from degradation (38–40). Our results are consistent with several recent reports demonstrating that proteasome inhibition increases hormone-induced activation of ERα (22). For example, inhibition of nuclear export with leptomycin B impairs E2-induced ERα proteasomal degradation and then increases ERα-mediated transcription (41). Increased proteasome-dependent ERα degradation and thus the depleted ERα level, induced by transforming growth factor-β1 (42) or hydroxamic acid analogue pan-HDAC inhibitors (43), abrogated its transcriptional activity, E2-driven DNA synthesis and cell growth in E2-responsive cancer cells. One possible explanation that could account for the phenomenon is that preventing ERα degradation effectively increases the number of functional receptors available to participate in transcriptional processes. It has been demonstrated that inhibiting receptor proteolysis with proteasome inhibitors increases the estrogen-binding capacity (25). Thus, receptors that evade proteolysis retain certain ERα functions, including ligand binding. Similar results have been reported with glucocorticoid receptor in embryonic hippocampal neurons, which show that activated receptors that are not targeted to proteasome retain transcriptional capacity (44). Studies of ERα in stable Chinese hamster ovary (45) and MCF-7 breast cancer cells (46) demonstrate that the magnitude of ERα transcriptional activity is directly related to the concentration of receptor. Thus, any regulatory mechanism that has the potential to elevate the concentration of functional ERα could similarly augment the transcriptional output of ERα.

Key protein stabilities in malignancy and their destructive destination by the ubiquitin-proteasome or related pathways will no doubt be an important focus of future endeavors. The necessity of proteasomal targeting in the delicate control of cellular growth is only beginning to unfold. Here, for the first time, we have demonstrated the inhibition of proteasome-de-
hPEBP4 Inhibits ERα Degradation via Src

26. Arnold, S. F., Obourn, J. D., Jaffe, H., and Notides, A. C. (1995) Mol. Endocrinol. 9, 24–33
27. Arnold, S. F., Melamed, M., Vorojeikina, D. P., Notides, A. C., and Sasson, S. (1997) Mol. Endocrinol. 11, 48–53
28. Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bonetempo, P., Nola, E., and Auricchio, F. (1996) EMBO J. 15, 1292–1300
29. Liu, J., Du, T., Yuan, Y., He, Y., Tan, Z., and Liu, Z. (2010) Mol. Cell. Biochem. 335, 29–35
30. Levin, E. R. (2005) Mol. Endocrinol. 19, 1951–1959
31. Accconcia, F., and Kumar, R. (2006) Cancer Lett. 238, 1–14
32. Marsaud, V., Gougelet, A., Maillard, S., and Renoir, J. M. (2003) Mol. Endocrinol. 17, 2013–2027
33. Shah, Y. M., and Rowan, B. G. (2005) Mol. Endocrinol. 19, 732–748
34. Fan, S., Wang, J., Yuan, R., Ma, Y., Meng, Q., Erdos, M. R., Pestell, R. G., Yuan, F., Auborn, K. J., Goldberg, I. D., and Rosen, E. M. (1999) Science 284, 1354–1356
35. Saji, S., Okumura, N., Eguchi, H., Nakashima, S., Suzuki, A., Toi, M., Nozawa, Y., Saji, S., and Hayashi, S. (2001) Biochem. Biophys. Res. Commun. 281, 259–265
36. Lonard, D. M., Nawaz, Z., Smith, C. L., and O'Malley, B. W. (2000) Mol. Cell 5, 939–948
37. Shen, T., Horwitz, K. B., and Lange, C. A. (2001) Mol. Cell. Biol. 21, 6122–6131
38. Alarid, E. T., Preisler-Mashek, M. T., and Solodin, N. M. (2003) Endocrinology 144, 3469–3476
39. Frasor, J., Stossi, F., Danes, J. M., Komn, B., Lyttle, C. R., and Katzenellenbogen, B. S. (2004) Cancer Res. 64, 1522–1533
40. Schreibhofer, D. A., Resnick, E. M., Lin, V. Y., and Shupnik, M. A. (2001) Endocrinology 142, 3361–3368
41. Calligé, M., Kieffer, I., and Richard-Foy, H. (2005) Mol. Cell. Biol. 25, 4349–4358
42. Petrel, T. A., and Brueggemeier, R. W. (2003) J. Cell. Biochem. 88, 181–190
43. Fiskus, W., Ren, Y., Mohapatra, A., Bali, P., Mandawat, A., Rao, R., Herger, B., Yang, Y., Atadja, P., Wu, J., and Btalla, K. (2007) Clin. Cancer Res. 13, 4882–4890
44. Wang, X., Pongrac, J. L., and DeFranco, D. B. (2002) Mol. Endocrinol. 16, 1887–1998
45. Webb, P., Lopez, G. N., Greene, G. L., Baxter, J. D., and Kushner, P. J. (1992) Mol. Endocrinol. 6, 157–167
46. Fowler, A. M., Solodin, N., Preisler-Mashek, M. T., Zhang, P., Lee, A. V., and Alarid, E. T. (2004) FASEB J. 18, 81–93