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Extracellular Signal-Regulated Kinase 7, a Regulator of Hormone-Dependent Estrogen Receptor Destruction

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Estrogen receptor alpha (ERα) degradation is regulated by ubiquitination, but the signaling pathways that modulate ERα turnover are unknown. We found that extracellular signal-regulated kinase 7 (ERK7) preferentially enhances the destruction of ERα but not the related androgen receptor. Loss of ERK7 was correlated with breast cancer progression, and all ERα-positive breast tumors had decreased ERK7 expression compared to that found in normal breast tissue. In human breast cells, a dominant-negative ERK7 mutant decreased the rate of endogenous ERα degradation >4-fold in the presence of hormone and potentiated estrogen responsiveness. ERK7 targets the ERα ligand-binding domain for destruction by enhancing its ubiquitination. Thus, ERK7 is a novel regulator of estrogen responsiveness through its control of ERα turnover.

Estrogen receptor alpha (ERα) belongs to the superfamily of ligand-activated transcription factors (7). This superfamily shares a common modular structure, which consists of an N-terminal region, a DNA-binding domain, and a ligand-binding domain. ERα regulates the expression of genes involved in growth and development and plays an important role in breast and endometrial cancers. The cellular response to estrogens in vivo is ERα limited (33), and a key mechanism in regulating ERα concentration is receptor degradation (2). Loss of ERα is associated with aggressive breast tumors and poor clinical outcome, and it is essential, therefore, to understand the molecular basis for the control of ERα turnover.

In response to estradiol, the rate of ERα degradation through ubiquitination and the 26S proteasome pathway is increased by an unknown mechanism (2, 20, 22, 35). The 26S proteasome pathway is the major pathway of regulated proteolysis in eukaryotes and is responsible for the destruction of ubiquitinated substrates (32). Ubiquitin is a 76-amino-acid protein that can be covalently attached to a lysine residue on the substrate through the action of an enzyme cascade involving E1, E2, and E3 enzymes. E1 activates ubiquitin and transfers it to E2 enzymes, which transfer ubiquitin to the substrate by themselves or in cooperation with E3 enzymes. E2 and E3 enzymes both contribute to target specificity.

Parallel studies on other members of the nuclear receptor superfamily have suggested a role for mitogen-activated protein kinase (MAPK) (also referred to as extracellular signal-regulated kinase 1/2 [ERK1/2]) in regulating receptor turnover. For example, MAPK(ERK1/2) phosphorylation of Ser-294 in the human progesterone receptor increases the progesterone receptor degradation rate (18). MAPK(ERK1/2) is also known to phosphorylate ERα, and we therefore investigated whether MAPK(ERK1/2) enhances ERα turnover (4, 14). MAPK(ERK1/2) belongs to a kinase subgroup, the activity of which is regulated by phosphorylation and/or dephosphorylation of threonine and tyrosine residues present in a Thr-Glu-Tyr (TEY) motif within the activation loop. Other members of this subgroup include ERK5 and ERK7. MAPK(ERK1/2) and ERK5 are both activated in response to growth factor signals (6, 15). However, ERK7 is a unique member of the TEY subgroup in that it is constitutively active and does not appear to be further activated by agents known to stimulate MAPK(ERK1/2) activity (1). Very little is known about ERK7, and its biological function has not been established.

We now show that ERK7 is a specific regulator of ERα degradation in human breast cells. ERK7 is expressed in normal human breast tissue, and loss of ERK7 is strongly correlated with breast cancer progression. Inhibition of ERK7 activity in human breast cells by a dominant-negative ERK7 mutant significantly enhances ERα levels in the presence of hormone and increases estrogen responsiveness. Our studies have therefore revealed the existence of a new signaling pathway impinging on the 26S proteasome machinery, in which ERK7 regulates hormone responsiveness in breast cells by controlling the rate of ERα degradation. Furthermore, the loss of this pathway appears to be correlated to the development of breast cancer.

MATERIALS AND METHODS

Reagents. The monoclonal antibodies, 12CA5 (antihemagglutinin [anti-HA]) and 9e10 (anti-MYC), were obtained from the University of Virginia Lymphocyte Culture Facility. The anti-active MAPK antibody (also called anti-pTEY antibody) was purchased from Promega. The cDNAs for human ERα, human IκBα, human androgen receptor (AR), and mouse SF1 were provided by P. Chambon (30), D. Ballard (3), M. Weber (9), and K. Parker (21), respectively. MEKc was provided by N. Ahn (25), ERK5 by J. Dixon (36), and HA-ubiquitin by D. Bohmann (31). The peptides to the C-terminal end of rat ERK7 (CRSA LGRLPLLPGPRA) and to the activation loop of rat ERK7 (CQALTEY) and

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the polyclonal antibodies, anti-ERK7 and anti-AL ERK7, were produced by Research Genetics.

Expression vectors. The cDNA for ERK7 was obtained from a rat testis cDNA library by using the PCR with primers specific to the published ERK7 sequence (1). The sequence was verified, and the cDNA was inserted in frame into pK3H (contains a triple HA tag) or PKR7-MYC (contains a MYC tag) (5) or modified pCMV-Tag5 (Stratagene). The vector pCMV-Tag5 (Stratagene) was modified by inserting the promoter and triple HA tag sequences from pKH3 into pCMV-Tag5. All ERα and ERK7 mutants were produced by using PCR, and the sequences were verified by the University of Virginia Biomolecular Research Facility. Both wild-type and mutant ERαs were subcloned into pSG5 (30).

Immunoblots. BHK cells were transfected in 10-mm-diameter dishes with calcium phosphate, 1 μg of wild-type or mutant ERα or FLAG-IκBα or HA-SF1 or HA-AR construct, and 5 μg of a construct encoding a wild-type or mutant kinase or vector control. At 8 h posttransfection, the cells were washed with phosphate-buffered saline and placed in Dulbecco's modified Eagle's medium (DMEM) with 5% charcoal-stripped fetal calf serum (FCS). After 1 h the cells were washed with phosphate-buffered saline and serum starved for 15 h, and either estradiol or vehicle was added 6 h before lysis with boiling sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (dithiothreitol [DTT]) (13).

For the rate experiments, transfected cells were trypsinized, aliquoted, and pretreated with 50 μM cycloheximide for 2 h in the absence of serum before the addition of estradiol. The cells were lysed at various times after estradiol treatment.

Breast lines were serum starved for 15 h before lysis with sample buffer (−DTT). Human tissue obtained from the University of Virginia Tissue Procurement Facility was pulverized in the presence of liquid N_2, and the powder was added to sample buffer (−DTT).

Total protein was determined by using DC protein assay (Bio-Rad), and 100 μg of protein was electrophoresed (DTT was added to each sample prior to loading) and transferred to nitrocellulose as described elsewhere (13). Immunoblots and densitometric analysis were as described (13), except that, for Fig. 6B, the 9e10 antibody was labeled directly with horseradish peroxidase.

Immunoprecipitations. BHK cells were transfected in 150-mm-diameter dishes with calcium phosphate and 2.5 μg of HA-ubiquitin construct and either 22.5 μg of MYC-ERK7 or vector control. Additionally, the cells were cotransfected with 2.5 μg of MYC-ERα(282-595) or additional vector control. The transfected cells were washed and then serum starved. Lysis and immunoprecipitation with 12CA5 were as described elsewhere (5).

Generation of stable clones. MCF-7 cells were maintained in DMEM with 5% charcoal-stripped FCS and 5% CO_2. They were transfected with Lipofectamine (Life Technologies) according to the manufacturer's directions with 6 μg of the modified pCMV-Tag5 vector with or without the K43A-ERK7 cDNA. Stable clones were obtained by using G418 (600 μg/ml) selection. Isolated colonies were cloned and propagated, and where appropriate the lines were analyzed for expression of HA-K43A-ERK7.

Transcriptional analysis. MCF-7 stables were transfected and assayed for luciferase and β-galactosidase as described previously (13).

Proliferation. Stable MCF-7 cells were seeded at 5×10^4 per well in a 24-well dish containing DMEM and 5% charcoal-stripped FCS with 600 μg of G418/ml. After 15 h the cells were treated with or without 1 μM ICI 182,780. At various times the cells were lysed and the amount of ATP was determined by CellTiter-Glo Luminescent Cell Viability Assay according to the manufacturer's protocol (Promega).

RESULTS

ERK7 specifically enhances ERα destruction. To test whether members of the MAPK(ERK1/2) family regulate ERα turnover, we determined whether any of the family members could influence the steady-state levels of ERα expression. ERα and the indicated kinases were coexpressed in baby hamster kidney (BHK) cells, and the expression levels of ERα and the kinases were determined by immunoblotting the lysates of the transfected cells. In these experiments BHK cells were chosen because they are easily transfected and support ERα-mediated transcription when provided with ERα cDNA (5). Ectopic expression of MAPK(ERK1/2) would not substantially increase cellular MAPK(ERK1/2) activity, because in order for MAPK(ERK1/2) to be active, it must be phosphorylated by MAPK kinase. Therefore, a constitutively active mutant of MAPK kinase (MEKc) was expressed to activate endogenous MAPK(ERK1/2). In this transient-transfection system, we did not observe any difference in the steady-state levels of ERα between vehicle and estradiol treatments in the vector control (Fig. 1A and B). However, in the vector control, the amount of ERα present in the more slowly migrating electrophoretic band increased in the presence of estradiol (Fig. 1A). It was previously determined that phosphorylation of Ser-118 in human ERα results in a reduced electrophoretic mobility (13). Activation of endogenous MAPK(ERK1/2) by MEKc increased the amount of ERα present in the more slowly migrating band, which can be most clearly observed by comparison with the vector control in the absence of estradiol. These data are consistent with observations that MAPK(ERK1/2) phosphorylates Ser-118 (4, 13). The anti-pTEY antibody (also referred to as the anti-active MAPK antibody) specifically recognizes the dually phosphorylated Thr and Tyr residues present in the activation loop of MAPK(ERK1/2) and ERK7 (1). Thus, as expected, the anti-pTEY immunoblot shows that the expression of HA-MEKc enhanced the activity of MAPK (ERK1/2) above that observed with the vector control. However, increasing MAPK(ERK1/2) activity did not alter ERα protein levels compared to those found in the vector control. Remarkably, however, the ectopic expression of ERK7 decreased the steady-state levels of ERα expression ~2-fold in the absence and ~3-fold in the presence of estradiol (Fig. 1B). The immunoblot with the anti-pTEY antibody demonstrates that similar levels of active ERK7 and endogenous MAPK (ERK1/2) were achieved. Expression of another member of the TEY kinase subgroup, ERK5, did not influence ERα levels (Fig. 1A), further demonstrating that a decrease in the steady-state level of ERα is a specific response to ERK7.

Steady-state levels reflect both the rates of synthesis and degradation. Therefore, to determine the effect of ERK7 on the rate of ERα destruction, we blocked protein synthesis by using the inhibitor, cycloheximide, and determined the amount of ERα by densitometry at various times after the addition of vehicle or estradiol. To eliminate transfection differences between time points, the BHK cells were distributed into the appropriate number of plates after being transfected. The rate data were fitted by using a single exponential decay and the rate constants were determined (Fig. 1C). Based on these rate constants, the half-life ($t_{1/2}$) for ERα in BHK cells is ~4 h and in the presence of estradiol is ~3 h (Fig. 1C and 2D). In BHK cells ectopic expression of ERK7 decreases the $t_{1/2}$ to ~2.5 and ~1.9 h in the absence and presence of estradiol, respectively. These data also demonstrate that the decrease in ERα levels in the presence of ERK7 is not a transfection artifact, because the rate data are independent of transfection efficiency.

To understand the role that the kinase activity of ERK7 plays in enhancing ERα destruction, we determined the ability of the mutant K43A-ERK7 to influence ERα steady-state levels. In this mutant the essential Lys-43, necessary for ATP hydrolysis, has been changed to Ala. K43A-ERK7 does not have catalytic activity in an in vitro kinase assay that uses myelin basic protein as a substrate (data not shown), in agreement with Abe et al. (1), who found that mutating Lys-43 to Arg destroyed ERK7 catalytic activity. Coexpression of ERα

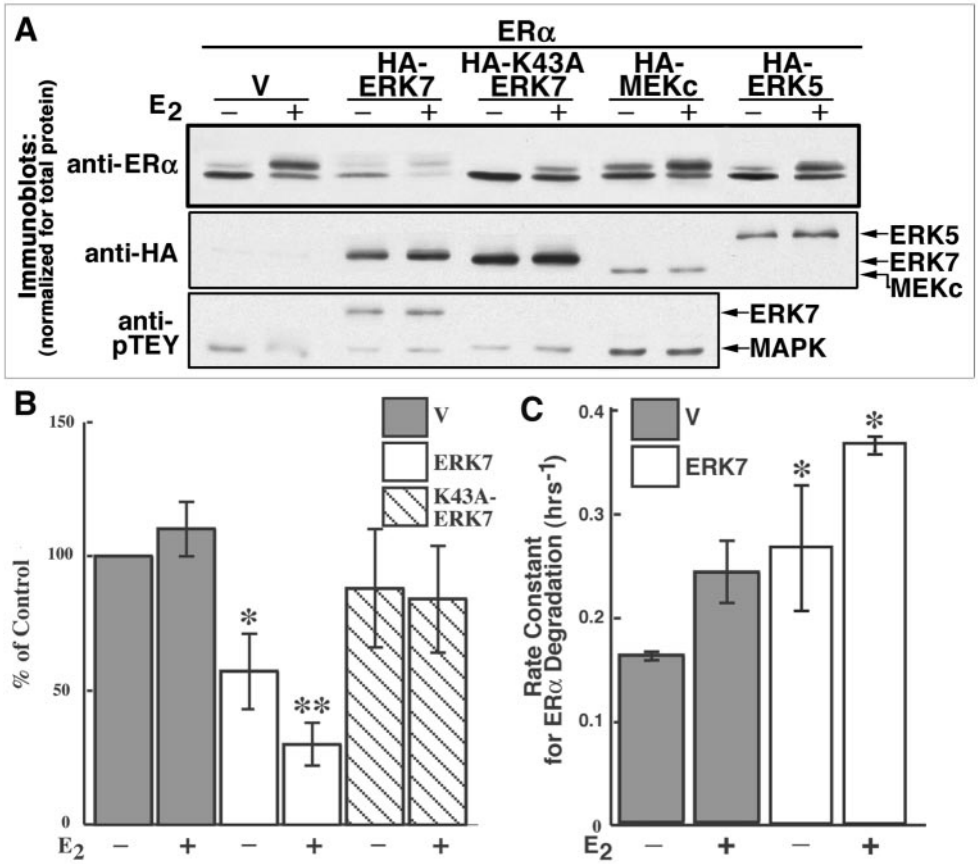

FIG. 1. Active ERK7 enhances ERα destruction. (A) BHK cells were cotransfected with vectors encoding ERα and either the indicated kinase or vector control (V). The transfected cells were serum starved and treated with or without 10 nM estradiol (E_2) for 6 h before addition of boiling sample buffer. Equal amounts of total protein were electrophoresed and immunoblotted. (B) Relative amounts of ERα were determined from the immunoblots by densitometry. The data are expressed as the percentage of ERα divided by ERα in the vector control in the absence of E_2. The means ± standard errors are shown for $n = 8$. *, $P < 0.05$; **, $P < 0.005$ (Student's t test); values were obtained by comparing ERα levels with coexpressed ERK7 to those obtained with the appropriate vector control. (C) BHK cells were cotransfected with vectors encoding ERα and either HA-ERK7 or vector control (V). The transfected cells were then aliquoted and pretreated with 50 μM cycloheximide for 2 h before the addition of vehicle or 10 nM E_2. Thereafter, at various time points over a 6-h period, the cells were lysed and immunoblotted and the relative amount of ERα was quantitated by densitometry. The rate data were fitted by using a single exponential decay, and the rate constants were determined. The means ± standard errors are shown for two separate experiments. *, $P < 0.05$ (Student's t test); value was obtained by comparing the rate constant to that of the appropriate control.

with kinase-dead ERK7 (HA-K43A-ERK7) did not significantly decrease ERα protein levels compared to those found in the vector control (Fig. 1A and B). Similar effects were also observed with a kinase-inactive ERK7 in which the Thr and Tyr present in the TEY phosphorylation motif were mutated to Ala (data not shown). Wild-type ERK7 and kinase-dead ERK7 were expressed to similar extents, but only wild-type ERK7 was able to decrease ERα levels. Therefore, these data demonstrate that the kinase activity of ERK7 is required to decrease ERα protein levels.

ERK7 specifically enhances ERα degradation. ERα is degraded through the 26S proteasome pathway, and one possible explanation for the observed decrease in ERα levels is that ERK7 enhances the rate of proteasome-mediated degradation. Therefore, we tested whether the competitive proteasome inhibitor MG132 could prevent ERK7 from decreasing ERα levels. In agreement with the results of Nawaz et al., addition of MG132 increased ERα protein levels in both the presence and absence of estradiol (Fig. 2A) (22). Treatment with MG132 also increased the amount of ERα coexpressed with ERK7 and increased the abundance of higher-molecular-weight forms of ERα induced by ERK7. In the presence of a proteasome inhibitor, the ubiquitinated product accumulates; thus, the higher-molecular-weight ERα forms are likely due to ubiquitination. Similar results were observed with another proteasome inhibitor, lactacystin (data not shown). It is not possible to determine by immunoblotting whether these forms are a result of ubiquitination because antiubiquitin antibodies are extremely insensitive. Additionally, our anti-ERα antibody does not immunoprecipitate the higher-molecular-weight ERα forms. These results suggest that ERK7 decreases the abundance of ERα by enhancing its degradation through the proteasome pathway.

To test the specificity of ERK7-mediated degradation, we determined whether ERK7 could enhance the destruction of IκBα, which is degraded by the 26S proteasome pathway (24).

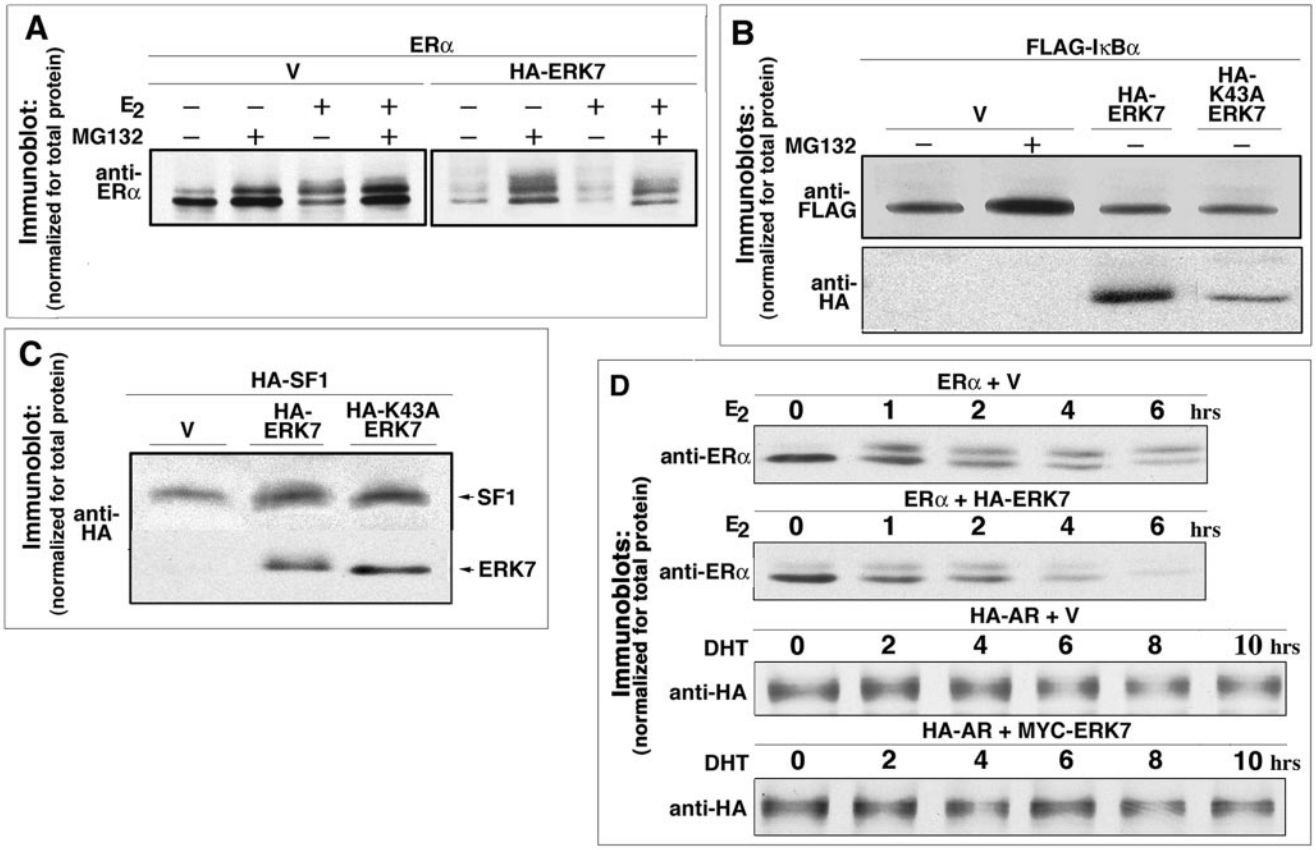

FIG. 2. ERK7 specifically enhances the degradation of ERα. (A) BHK cells were cotransfected with vectors encoding ERα and either HA-ERK7 or vector control (V). The serum-starved, transfected cells were pretreated with or without 25 μM MG132 for 2 h before addition of vehicle or E_2 and were then lysed after 6 h, and equal amounts of protein were electrophoresed and immunoblotted. (B) BHK cells were cotransfected with vectors encoding FLAG-IκBα and HA-ERK7 or HA-K43A-ERK7 or vector control (V). The serum-starved, transfected cells were treated with or without MG132 for 8 h and lysed, and equal amounts of protein were electrophoresed and immunoblotted. (C) BHK cells were cotransfected with vectors encoding HA-SF1 and HA-K43A-ERK7 or HA-ERK7 or vector control (V). The serum-starved, transfected cells were lysed, and equal amounts of protein were electrophoresed and immunoblotted. (D) BHK cells were cotransfected with vectors encoding ERα or HA-AR and HA-ERK7 or MYC-ERK7 or vector control (V). The transfected cells were then aliquoted and pretreated with 50 μM cycloheximide for 2 h before the addition of 10 nM E_2 or 100 nM dihydroxytestosterone (DHT). Thereafter, at various times after ligand addition the cells were lysed and immunoblotted.

ERK7 did not reduce the protein levels of IκBα in comparison to the vector control (Fig. 2B). These results support the hypothesis that ERK7 is a specific regulator and is not a general activator of the ubiquitination and proteasome machinery.

To determine whether ERK7 can trigger the destruction of other nuclear receptor superfamily members, we used steroidogenic factor I (SF1), a distant, evolutionarily related relative of ERα (19). ERK7 had no effect on the protein levels of SF1 compared to the vector control (Fig. 2C). These results suggest that ERK7 stimulates the degradation of only a subset of the nuclear receptor superfamily, which includes ERα.

To further define the nuclear receptors that are targets of ERK7, we tested the ability of ERK7 to enhance the rate of degradation of AR, a close relative of ERα. The rate of degradation was determined in the presence of cycloheximide in a similar manner to that performed with ERα, except that dihydroxytestosterone was used. Remarkably, ERK7 did not enhance the degradation of the AR (Fig. 2D). Taken together, these data suggest that ERK7 specifically regulates ERα turnover.

ERK7 is highly expressed in normal human breast cells. To understand the importance of ERK7 in regulating estrogen action, we determined whether ERK7 was expressed in breast cells, an estrogen target tissue. We generated a polyclonal antibody to the extreme C terminus of rat ERK7, which was able to specifically detect ERK7 but not a truncated mutant of ERK7 in lysates from transfected BHK cells (Fig. 3A, left). Note that deletion of the C-terminal tail of ERK7 also inhibits its constitutive kinase activity and that the truncation is therefore not detected by the anti-pTEY antibody. The anti-ERK7 antibody was used to immunoblot lysates of MCF-10A, a normal breast cell line, and the breast cancer cell lines MCF-7 and MDA-MB-231 (Fig. 3B). A band of ~60 kDa was strongly detected by the anti-ERK7 antibody in MCF-10A cells, and this molecular mass is in agreement with the calculated molecular mass based on the rat ERK7 cDNA. The ~60-kDa band recognized by anti-ERK7 colocalized with a band recognized by the anti-pTEY antibody (Fig. 3B). Together, these data strongly suggest that the ~60 kDa-band observed in normal breast cells is human ERK7. It is not particularly surpris-

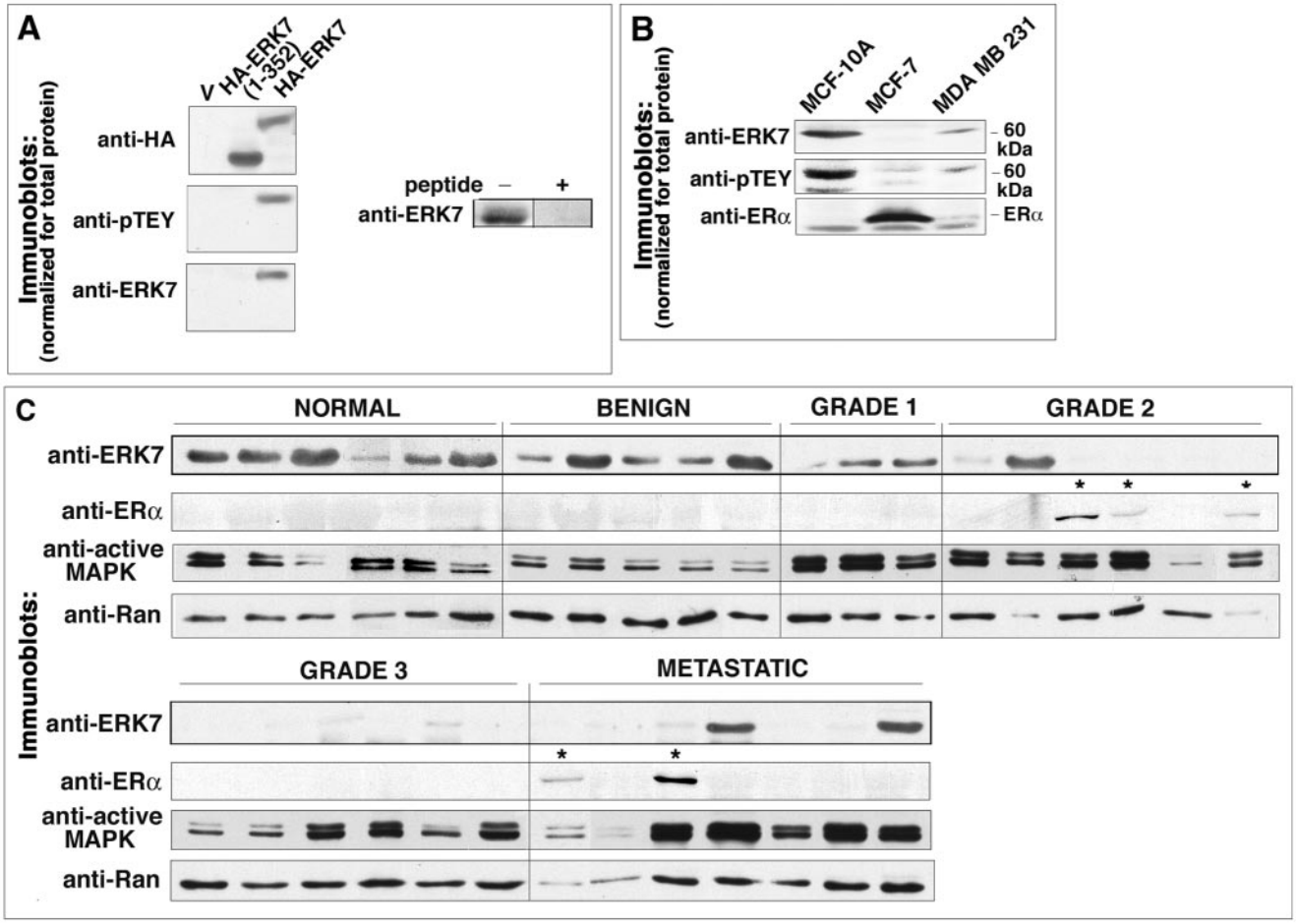

FIG. 3. ERK7 is expressed in normal human breast cells. (A) In the left panel BHK cells were transfected with vectors encoding an HA-tagged wild type or a deletion mutant of ERK7 or vector control (V). The serum-starved, transfected cells were lysed, and equivalent amounts of protein were electrophoresed and immunoblotted. In the right panel normal human breast tissue was electrophoresed and immunoblotted with anti-ERK7 in the presence (+) or absence (−) of the antigenic peptide (200 μM) used to produce anti-ERK7 (p). (B) Equivalent amounts of lysate from serum-starved MCF-10A, MCF-7, and MDA-MB-231 cells were electrophoresed and immunoblotted. (C) Normal human breast tissue, benign tumor tissue, and breast cancer tissue samples were solubilized, normalized for Ran expression, electrophoresed, and immunoblotted. The tumor grade was obtained from the pathologist's report. ERα-positive samples are indicated by an asterisk.

ing that the antibody raised to rat ERK7 recognizes human ERK7, because a number of kinases are virtually identical in their amino acid sequences between rats and humans, e.g., ERK2.

The MCF-10A cell line has higher levels of ERK7 than the MCF-7 and MDA-MB-231 cell lines. The amount of ERα in the different cell lines is inversely correlated with the amount of ERK7 (Fig. 3B). The amount of ERα is extremely small in MCF-10A and can be detected only by immunoblotting anti-ERα immunoprecipitations (data not shown). These results are consistent with the idea that ERK7 regulates ERα turnover, because the MCF-10A cell line has low levels of ERα and because the MCF-7 cell line has high levels of ERα. The MDA-MB-231 cell line represents an intermediate between MCF-10A and MCF-7.

To further understand the physiological significance of ERK7 in regulating estrogen action, we determined the expression levels of ERK7 in normal human breast tissue and breast cancer tissue (Fig. 3C). Detection of the ~60-kDa band in normal human breast tissue was blocked by preincubating the anti-ERK7 antibody with the antigenic peptide (Fig. 3A, right). In total we examined 13 normal tissues, 5 benign tumors, and 66 breast tumors, and the results shown are representative. The samples are grouped by their tumor grade from the least to the most aggressive, according to the pathologist's report. The tissue samples were normalized to each other by immunoblotting for Ran, a housekeeping protein whose expression level is not known to change with any disease state. ERK7 was expressed in all of the normal and benign tissue samples, approximately half of the grade 1 samples, ~20% of the grade 2 tumors, none of the grade 3 tumors, and ~30% of the metastatic tumors (Fig. 3C). Thus, loss of ERK7 is correlated with breast cancer progression. These samples were also analyzed for active MAPK(ERK1/2), and in agreement with the literature (26, 27), we observed that MAPK(ERK1/2) activity was generally higher in breast cancers than in the normal or benign tumor samples. But, importantly, we did not observe a correlation between MAPK(ERK1/2) activity and loss of

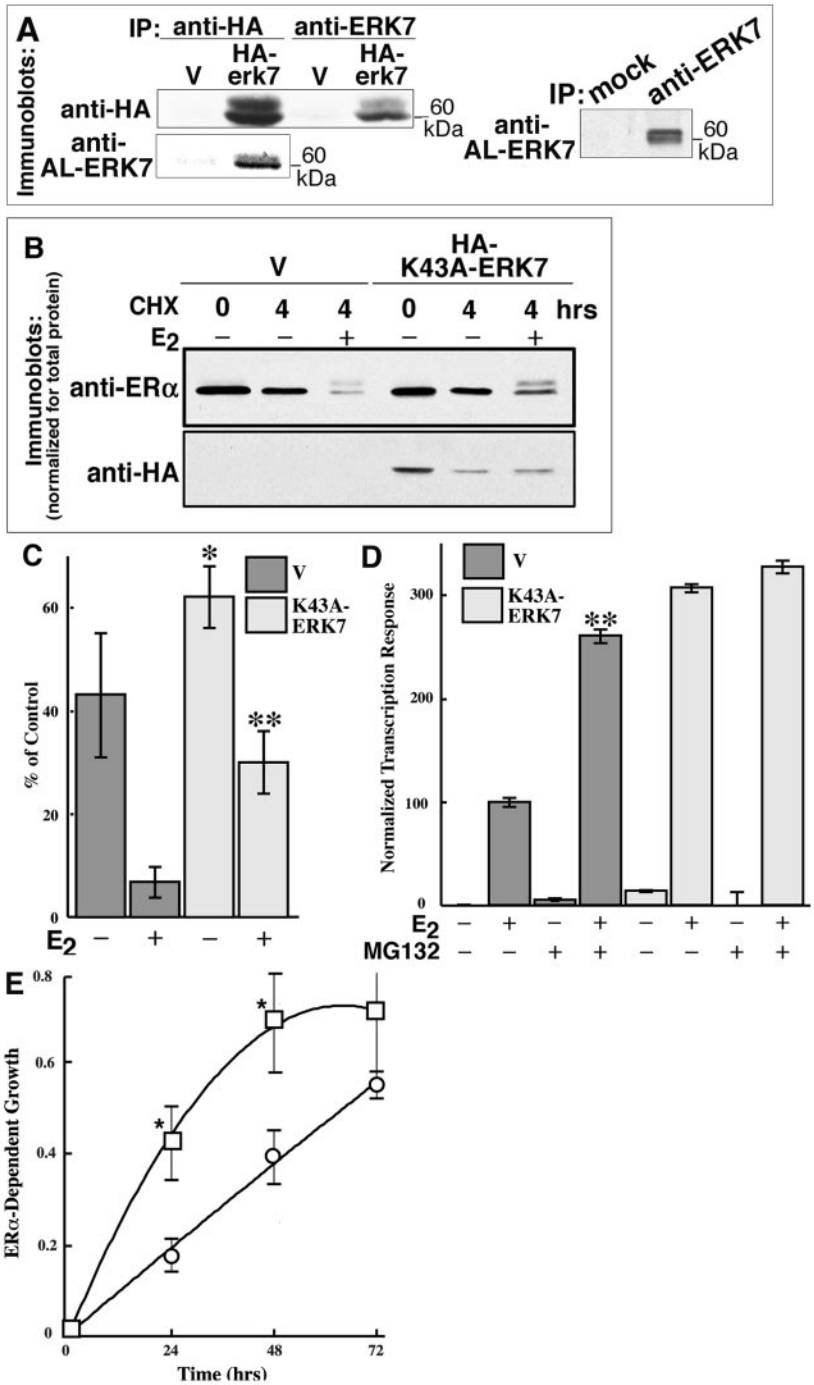

FIG. 4. ERK7 regulates endogenous ERα degradation in human mammary cells. (A) In the left panel BHK cells were transfected with vector encoding HA-ERK7 or the vector control (V). The serum-starved, transfected cells were lysed, and the HA-ERK7 was immunoprecipitated (IP) with either anti-HA or anti-ERK7 antibodies. The IPs were electrophoresed and immunoblotted. In the right panel serum-starved MCF-7 cells were lysed and either immunoglobulin G or anti-ERK7 antibody was added to the lysate. The immunoprecipitates were electrophoresed and immunoblotted. (B) Serum-starved MCF-7 stable lines expressing either vector alone (V) or HA-K43A-ERK7 were immediately lysed after the addition of 50 μM cycloheximide (CHX) or after a 4-h pretreatment with CHX with or without 10 nM E_2. Equivalent amounts of protein were electrophoresed and immunoblotted. (C) Relative ERα levels were determined by densitometry, and the data were expressed for each stable line as the percentage of ERα after incubation with CHX divided by ERα before incubation with CHX. The means ± standard errors for two separate experiments are shown. *, $P < 0.05$; **, $P < 0.005$ (Student's t test); values were obtained by comparing the response obtained with the lines expressing K43A-ERK7 to the appropriately treated control. (D) MCF-7 stables were cotransfected with an ERE-regulated luciferase reporter and β-galactosidase expression vectors. The serum-starved, transfected cells were treated with or without E_2 and MG132. The luciferase and β-galactosidase activity was determined 24 h after the indicated treatments. The luciferase data were normalized to β-galactosidase activity to control for differences in transfection efficiency. To facilitate comparison between different experiments, the data were normalized so that, in the vector control, the response to vehicle addition was 0 and the response to E_2 was 100. The means ± standard errors are shown ($n = 3$), each done

ERK7. These results demonstrate that the loss of ERK7 is not merely a reflection of a general decrease in the levels of various members of the MAPK(ERK1/2) family. Also in agreement with the literature (29), we observed that normal human breast mammary epithelial cells have very low levels of ERα, which is consistent with our observations that normal breast tissue has significant amounts of ERK7. Additionally, cancer tissues that had detectable amounts of ERα had undetectable levels of ERK7 expression. Thus, loss of ERK7 correlates with increased ERα levels. These data further suggest that ERK7 is an important regulator of estrogen action in the breast.

ERK7 regulates hormone responsiveness in human breast cells. The concentration of ERα is limiting for estrogen responsiveness in vivo, and ERα synthesis and degradation, therefore, play a pivotal role in controlling the expression of ERα-regulated genes (33). Taken together, our results argue that ERK7 may regulate hormone responsiveness in cells that endogenously express ERα. To test this hypothesis, we attempted to produce MCF-7 clones that stably expressed HA-ERK7; however, we found that the majority of the ERK7 was not active (data not shown). These results suggest that ERK7 activity is regulated differently in breast cells from how it is regulated in BHK cells, in which ~50% of ERK7 is active. Kinase-dead mutants are often able to act as dominant negatives; therefore, we produced MCF-7 clones that stably expressed K43A-ERK7. We hypothesized that MCF-7 cells contained extremely low levels of ERK7 and that we could inhibit its ability to degrade ERα by using K43A-ERK7. In MCF-7 lysates we observed a faint band at ~60 kDa upon extended exposure of the anti-ERK7 immunoblot (data not shown). The anti-ERK7 antibody is able to immunoprecipitate HA-ERK7 from lysates of transfected cells (Fig. 4A, left); therefore, we used this antibody to immunoprecipitate ERK7 from MCF-7 lysates. ERK7 was detected in the immunoprecipitate by use of another polyclonal antibody to ERK7 that recognizes residues in the activation loop of ERK7 (anti-AL ERK7) (Fig. 4A, right). The anti-AL ERK7 recognizes both the active and inactive forms of ERK7 (Fig. 4A, left). Thus, these results demonstrate that MCF-7 cells do contain ERK7.

In the K43A-ERK7 stable cell lines, addition of cycloheximide decreased the degradation of ERα by ~1.5-fold and >4-fold in the absence and presence of estradiol, respectively, compared to that in the stable vector lines (Fig. 4B and C). Thus, K43A-ERK7 is more effective at inhibiting the degradation of the hormone-bound receptor than that of the unbound receptor. Over the 4-h course of cycloheximide treatment, the level of K43A-ERK7 decreased significantly, which suggests that ERK7 is turned over rapidly in MCF-7 cells. It is likely that the effects on ERα degradation would have been much more dramatic had the levels of K43A-ERK7 remained constant over the time course of the experiment. These results suggest that K43A-ERK7 is able to act as a dominant negative in MCF-7 cells by inhibiting the action of endogenous ERK7.

To further test whether ERK7 is an important regulator of estrogen action, we determined the effect of K43A-ERK7 on ERα-regulated transcription. The MCF-7 stable lines were cotransfected with a vector encoding the luciferase reporter under the control of estrogen-responsive elements (ERE), plus a control vector encoding β-galactosidase. Cells expressing K43A-ERK7 had an ~3-fold-greater transcriptional response to estradiol than the control cells (Fig. 4D). MG132 also increased the transcriptional response of the control cells by ~3-fold. However, the transcriptional response in the K43A-ERK7 cells was not affected by MG132. These results suggest that K43A-ERK7 and MG132 influence ERα-mediated transcription through a similar mechanism, by inhibiting the ERα degradation rate. In our experiments, a TATA box and a dual ERE regulate luciferase reporter expression. Therefore, we next tested whether ERK7 also regulates ERα-regulated transcription of endogenous genes in MCF-7 cells. As a readout for broad responses to ERα, we used the ability of estrogen to stimulate MCF-7 cell proliferation, because the proliferation response is known to be regulated by ERα transcriptional activation (8, 28). It is likely that ERK7 regulates the activity of molecules in addition to ERα and that these other molecules may influence proliferation. Therefore, to examine only the effects of K43A-ERK7 on ERα-mediated proliferation, we determined growth in the presence of vehicle or ICI 182,780, a pure estrogen antagonist (10). To examine only the ERα-dependent growth, we subtracted the growth in the presence of ICI 182,780 from that obtained with the vehicle. K43A-ERK7 enhanced the rate of ERα-dependent proliferation ~2-fold (Fig. 4E). These data suggest that ERK7 regulates ERα-mediated transcription from both simple and complex promoters. Furthermore, the ability of K43A-ERK7 to promote proliferation suggests that ERK7 regulation of the ERα degradation rate is important in determining estrogen responsiveness.

ERK7 phosphorylation of ERα does not influence ERα stability. Because turnover is commonly regulated by phosphorylation of the target protein, it seemed possible that ERK7 regulates ERα degradation by direct phosphorylation (17). ERK7 is most likely a proline-directed serine/threonine kinase because the catalytic domain is highly related to MAPK (ERK1/2) (1); thus, there are four putative ERK7 phosphorylation sites in ERα, Ser-104, Ser-106, Ser-118, and Ser-294. It was previously determined that Ser-118 is the major site of phosphorylation in response to estradiol binding (12); therefore, we determined whether ERK7 could phosphorylate ERα in vivo. Lysates from cells transfected with wild-type ERα or S118A-ERα in the presence or absence of ERK7 were normalized for total ERα and were immunoblotted with anti-ERα. The lysates were normalized to ERα rather than total

in quadruplicate. **, $P < 0.005$ (Student's t test); this value was obtained by comparing the E_2 response obtained with the vector control in the presence of MG132 to that obtained in its absence. (E) Stable MCF-7 lines were treated with or without 1 μM ICI 182,780. At various times the cells were lysed and growth was determined. ERα-dependent growth was obtained by subtracting the growth in ICI 182,780 from the growth obtained in vehicle control. The results are taken from two experiments in which each time point was determined in triplicate and from two independent lines. □, K43A-ERK7; ○, V. *, $P < 0.05$ (Student's t test); value was obtained by comparing the response obtained with the stable lines expressing HA-K43A-ERK7 to that found with the appropriately treated control.

protein so that we could directly compare the intensities of the various ERα bands between the ERK7 and vector control samples. The appearance of Ser-118 phosphorylation can be observed as a sharp band, and ERK7 enhances the amount of this phosphorylation in both the absence and presence of estradiol (Fig. 5A). The kinase-dead mutant, K43A-ERK7, diminishes the intensity of this more slowly migrating ERα band (Fig. 1A). Taken together, these data suggest that ERK7 can regulate either directly or indirectly the level of Ser-118 phosphorylation. ERK7 also increases the number of other higher-molecular-weight ERα forms that are observed as diffuse bands both in wild-type ERα and the S118A-ERα mutant. The identity of these bands will be discussed later.

To determine the role that ERα phosphorylation may play in ERK7-mediated ERα destruction, we determined the ability of ERK7 to enhance the rate of S118A-ERα degradation in the presence of cycloheximide. ERK7 was able to enhance the degradation of S118A-ERα with a $t_{1/2}$ similar to that observed with the wild-type ERα (Fig. 5B). We also tested whether ERK7 could enhance the degradation of mutant ERαs that contained mutations in the other putative ERK7 phosphorylation, i.e., Ser-104, Ser-106, and Ser-294. There were no significant differences in the ability of ERK7 to decrease wild-type ERα protein levels compared to levels of any of the mutants (data not shown). We also found that ERK7 did not enhance the degradation of a deletion mutant of ERα lacking the ligand-binding domain (ERα[1-282]) (Fig. 6A). These data are consistent with those obtained with MEKc (Fig. 1), in which enhanced Ser-118 phosphorylation by MAPK(ERK1/2) did not influence ERα levels. Thus, although ERK7 may enhance Ser-118 phosphorylation, it seems that mechanisms other than ERα phosphorylation are important in targeting ERα for destruction.

The ligand-binding domain targets ERα for ERK7-mediated destruction. ERK7 targets the wild-type ERα for degradation but not a deletion mutant lacking the ligand-binding domain. Therefore, we determined whether ERK7 enhanced the destruction of the ligand-binding domain. In agreement with the literature (20), we observed that estradiol enhanced the level of ERα(283-595) coexpressed with the vector control (Fig. 6A). Nonetheless, ERK7 was able to decrease the expression level of ERα(283-595) in the presence and absence of estradiol. These results suggest that the ligand-binding domain plays an important role in the ability of ERK7 to regulate ERα protein levels.

Inhibitors of the 26S proteasome pathway are able to decrease ERK7-mediated ERα degradation (Fig. 2A), and ERK7 enhances the formation of higher-molecular-weight ERα forms (Fig. 2A and 5A). Therefore, we suspected that these high-molecular-weight forms consist of ubiquitinated ERα. However, as mentioned before, it is not possible to directly determine whether these higher-molecular-weight forms are the result of ubiquitination. Therefore, to test our hypothesis that ERK7 enhances ERα ubiquitation, BHK cells were cotransfected with constructs encoding HA-ubiquitin plus either MYC-tagged ERα(283-595) or vector control. Additionally, the cells were transfected with either MYC-tagged ERK7 (MYC-ERK7) or vector control and were treated with MG132. To isolate ubiquitinated proteins, lysates were immunoprecipitated with anti-HA antibody and were then immunoblotted with either anti-MYC or Ab10, an antibody to the ligand-binding domain of ERα. The amount of ERα(283-595) containing HA-ubiquitin was greatly increased in the presence of ERK7 compared to that found with the vector control (Fig. 6B). The first arrow indicates a band that is at a molecular weight consistent with the addition of a single ubiquitin to MYC-ERα(283-595), whereas the other bands are consistent with polyubiquitination. The results with Ab10 were similar to those shown with the α-MYC antibody (data not shown). These results support the hypothesis that ERK7 enhances the level of ERα ubiquitination.

DISCUSSION

Human ERK7 was identified based on the ability of two different antibodies to recognize a protein of a molecular weight similar to that predicted for rat ERK7. Support for the physiological importance of ERK7 is provided by our observations that ERK7 is expressed in human breast tissue, an estrogen target, and that there is a decrease in ERK7 expression during breast cancer progression. Furthermore, in all tumor samples that are ERα positive, the expression of ERK7 has been lost. This inverse correlation between ERα and ERK7 expression levels was also observed in human breast cell lines. Further evidence for the physiological importance of ERK7 is provided by our observations that a dominant-negative ERK7 mutant is able to regulate hormone responsiveness in breast cells as measured by transcription and proliferation. A dominant-negative ERK7 mutant was able to enhance ERα-mediated transcription to the same extent as treatment with the proteasome inhibitor, MG132, which indicates that endogenous ERK7 is the predominant pathway of ERα degradation in breast cells.

Our results support a model in which ERK7 enhances hormone-dependent ERα destruction through the 26S proteasome pathway by increasing ERα ubiquitination. This model is supported by observations that a dominant-negative ERK7 mutant preferentially inhibits degradation of the hormone-bound ERα compared to that of unbound ERα. ERK7 enhances the level of ubiquitination of the ligand-binding domain, and this domain is required for ERK7-mediated destruction. In response to hormone binding, it is known that the ligand-binding domain undergoes a conformational change (23) and presumably this conformational change targets hormone-bound ERα for destruction by ERK7.

It has been previously shown that, in response to hormone, the phosphorylation of Ser-118 increases (12). ERK7 enhances Ser-118 phosphorylation in vivo, but this phosphorylation is not important for ERK7-mediated degradation, because ERK7 is able to enhance the rate of degradation of ERα and Ser-118–ERα to the same extent. We were also unable to find any evidence that mutation of other putative ERK7 phosphorylation sites influenced ERK7-mediated ERα degradation. It seems unlikely that phosphorylation of the ligand-binding domain plays a role in regulating turnover, because enhanced phosphorylation in the ligand-binding domain during use of [^{32}P]orthophosphate labeling and microsequencing of the radiolabeled ERα peptides in the absence or presence of lactacystin has not been observed (12, 13; data not shown).

There are several cases known in which the destruction of

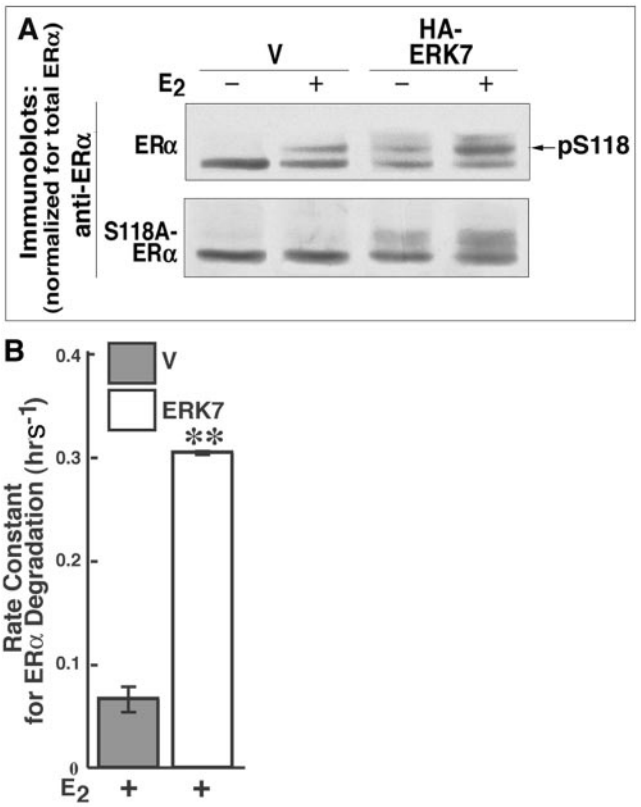

FIG. 5. Ser-118 phosphorylation does not influence ERα turnover. (A) BHK cells were cotransfected with vectors encoding ERα or S118A-ERα and HA-ERK7 or vector control (V). The serum-starved, transfected cells were treated with or without 10 nM E_2 for 6 h before lysis. Lysates containing equivalent amounts of ERα were electrophoresed and immunoblotted. (B) BHK cells were cotransfected with vectors encoding S118-ERα and either HA-ERK7 or vector control (V). The transfected cells were then aliquoted and pretreated with 50 μM cycloheximide for 2 h before the addition of 10 nM E_2. At various time points after E_2 addition, the cells were lysed and immunoblotted and the relative amount of S118A-ERα was quantitated by densitometry. The rate constants were determined as described in the Fig. 1C legend, and the data shown are the means ± standard errors for two separate experiments.

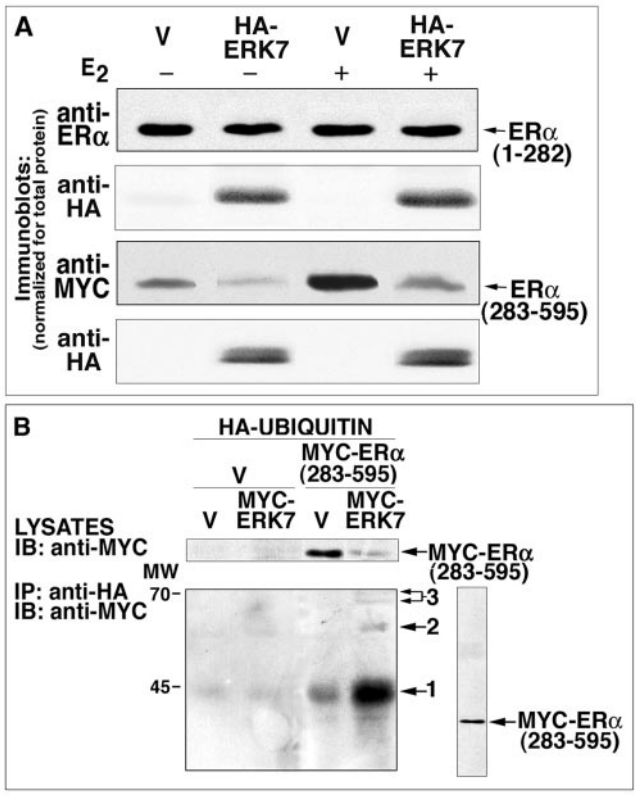

FIG. 6. ERK7 enhances the ubiquitination of the ERα ligand-binding domain. (A) BHK cells were cotransfected with vectors encoding a deletion mutant of ERα and either HA-ERK7 or vector control (V). The serum-starved, transfected cells were treated with or without 10 nM E_2 for 6 h and were then lysed. Equal amounts of total protein were electrophoresed and immunoblotted. (B) BHK cells were transfected with a vector encoding HA-ubiquitin and either MYC-ERK7 or vector control (V). They were also transfected with either additional vector control or a vector encoding MYC-ERα(283-595). The transfected cells were pretreated with MG132 for 2 h before lysis. Immunoprecipitations (IPs) were performed by using anti-HA antibody, and the IPs were electrophoresed and immunoblotted (IB).

specific targets is regulated by kinases. The kinases phosphorylate a residue within a specific sequence context, and this phosphorylation results in the interaction with particular E2 and E3 enzymes. For example, glycogen synthase kinase 3β phosphorylates β-catenin and IκB kinase phosphorylates IκBα, which regulates their destruction through the Skp1-Cullin-F-box complex (11, 16, 24). There is also evidence that the destruction of some members of the nuclear steroid receptor superfamily is regulated by their phosphorylation (18, 34). However, our discovery of ERK7-mediated destruction of ERα is mechanistically distinct. One possible mechanism is that ERK7 phosphorylates a component of the ubiquitin machinery, which increases its catalytic activity or affinity for ERα. Another plausible mechanism is that ERK7 phosphorylates an ERα-interacting protein, which exposes a surface on ERα that targets it for destruction. Although we have not elucidated all the components of the signaling pathway leading from ERK7 to enhanced ERα degradation, we have provided the first biologically relevant function for ERK7.

Remarkably, ERK7 expression is lost during breast cancer progression. Normal breast epithelial cells have extremely low levels of ERα and are considered to be ERα negative (29). Hormone-responsive breast cancers express 10-fold-higher or greater ERα levels, but as the cancer becomes more aggressive, ERα expression is frequently lost. In our studies all the ERα-positive tumors also had lost ERK7 expression, and it may be that the increase in ERα levels in hormone-responsive breast cancers is due to decreased ERK7 expression. However, not all tumors that had lost ERK7 expression were ERα positive, suggesting that other factors in addition to ERK7 are involved in regulating ERα levels. It may be that during tumor progression ERK7 downstream effectors have been inappropriately activated, resulting in reduced ERα levels even in the absence of ERK7. We suggest that ERK7 is important in maintaining the homeostasis of a normal breast cell and that with high frequency ERK7 is lost at an early step in breast cancer progression.

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