Proteasome-Mediated Proteolysis of Estrogen Receptor: A Novel Component in Autologous Down-Regulation

Elaine T. Alarid, Natalie Bakopoulos, and Natalia Solodin

The Department of Physiology
University of Wisconsin-Madison
Madison, Wisconsin 53706

Regulation of estrogen receptor (ER) concentration is a key component in limiting estrogen responsiveness in target cells. Yet the mechanisms governing ER concentration in the lactotrope cells of the anterior pituitary, a major site of estrogen action, are undetermined. In this study, we used a lactotrope cell line, PR1, to explore regulation of ER protein by estrogen. Estrogen treatment resulted in an approximate 60% decrease in ER steady state protein levels. Suprisingly, the decline in ER protein was apparent within 1 h of estrogen treatment and occurred in the absence of protein synthesis and transcription. Direct regulation of ER protein was further confirmed by pulse chase analysis, which showed that ER protein half-life was shortened from greater than 3 h to 1 h in the presence of estrogen. The estrogen-induced degradation of ER protein could be prevented by pretreatment with peptide aldehyde inhibitors of proteasome protease whereas inhibitors of calpain and lysosomal proteases were ineffective. Inhibition of proteasome activity maintained ER protein at a level equivalent to control cells not stimulated with estrogen but increased estrogen-binding activity by 1.75-fold. Proteolytic regulation of ER by the proteasome is not limited to pituitary lactotrope cells but is also operational in MCF-7 breast cancer cells, suggesting that this may be a common regulatory pathway used by estrogen. These studies describe a nongenomic action of estrogen that involves nuclear ER: rapid proteolysis of ER protein via a proteasome-mediated pathway. (Molecular Endocrinology 13: 1522–1534, 1999)

INTRODUCTION

Steroid hormone action is, in large part, controlled by cellular receptor concentration. Unlike peptide hormone receptors, which are single components of complex signal transduction cascades, steroid receptors transmit signal directly to DNA. As ligand-activated transcription factors, they bind regulatory elements in responsive genes and, along with coactivators or corepressors, control transcription. Although other proteins are involved, the receptor is the limiting factor that dictates the magnitude of the steroid response. In cell lines engineered to overexpress glucocorticoid receptor (GR), there is a linear relationship between the amount of GR and transcriptional activation of target genes (1). Similar studies of estrogen receptor (ER) indicate that physiological levels of ER limit estrogen transcriptional activity well below the cellular capacity to respond to estrogen (2). This pivotal role of steroid receptors makes them a focal point in regulating steroid hormone function.

The level of steroid receptors in cells changes with varying physiological states. In most cases, the primary endocrine regulator is the ligand itself. In an autoregulatory feedback loop, estrogen induces a decline in both ER protein and mRNA (3–13). Several mechanisms have been proposed to explain how estrogen controls ER levels, most of which focus on regulation at the level of RNA. Studies using Rat-1 fibroblasts stably transformed with ER (Rat 1+ER) (7) and MCF-7 breast cancer cells (11, 12) support both transcriptional and posttranscriptional mechanisms. The focus on transcriptional mechanisms is based on the assumption that decreased protein concentration is largely a consequence of decreased steady state levels of mRNA.

ER regulation by estrogen has been documented in a number of systems, yet little is known about mechanisms governing ER regulation in estrogen target tissues outside of breast cancer cell lines and uterus. To examine ER regulation in the pituitary, we took advantage of the recent derivation of a lactotrope cell line named PR1. The PR1 cell line was derived from an estrogen-induced lactotrope hyperplasia in F344 rats (14). It exhibits unique sensitivity to estrogen with a high affinity for estradiol [dissociation constant (K_d) = 10^{-11} M (15)]. Like MCF-7 cells and other model systems, we observed that estrogen induces a decrease

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in ER protein levels in PR1 cells. However, within the first 1–2 h, ER protein levels decrease without a concomitant decline in mRNA levels. The rapid loss of ER protein in the absence of changes in ER mRNA suggested that ER protein may be regulated independently of transcription. Utilizing a short time frame of estrogen exposure, we are able to isolate changes in ER protein levels away from changes in RNA. This permits the exploration of regulatory mechanisms directly controlling ER protein. Here we report that estrogen stimulates degradation of ER protein via a proteasome-mediated proteolytic mechanism.

RESULTS

Estrogen Action on ER in Pituitary Lactotrope Cells

Study of the regulation of ER in the pituitary has been hampered by the lack of a model system that possesses endogenous ER and that exhibits robust responses to estrogen. PR1 cells, like the hyperplasia from which they were derived, are extremely sensitive to estrogen and are stimulated to proliferate at doses of estradiol as low as 10^{-14} M (15). We capitalized on the hypersensitivity of these cells to examine the mechanism(s) governing estrogen regulation of ER in lactotrope cells. Total ER protein levels were monitored by Western blot analysis of whole-cell extracts. Time course (Fig. 1, A and B) and dose response (Fig. 1, C and D) experiments indicate that 10^{-10} M 17β-estradiol (E_2) is sufficient to elicit an approximate 50% decrease in ER protein levels at 1 and 2 h. These results were confirmed using primary anti-ER antibodies directed against the hinge and C-terminal regions to demonstrate that the loss of signal was not due to epitope masking (data not shown). Moreover, the identical results with different antibodies suggest that this decline is representative of total ER protein and not an epitope-specific pool. To further verify that the decrease in protein was specific to ER, blots were reprobed with antibody directed against the ubiquitous protein, IκBα, which is not regulated by estrogen. IκBα protein levels were unchanged in the presence and absence of E_2 and serve as a loading control for total protein content (Fig. 1A, *lower panel*).

Analysis of autologous down-regulation of ER protein in MCF-7 and Rat 1+ER cells suggested that estrogen reduces ER primarily by decreasing steady state ER mRNA levels (3, 7–13). In contrast, Northern blot analysis of ER mRNA in PR1 cells shows that mRNA levels do not change within 2 h of E_2 treatment (see below, Fig. 5, lanes 1 and 2). These results suggested that estrogen may induce a rapid decrease in ER protein that is independent of ER synthesis. To test this possibility, ER protein levels were examined in the presence of inhibitors of protein synthesis and transcription. Figure 2A shows that halting *de novo* protein synthesis with a series of inhibitors can decrease ER protein levels relative to untreated controls. However, in no case was the decline in ER levels equivalent to that induced by estrogen. Furthermore, the addition of inhibitors of either protein synthesis (Fig. 2A) or transcription (Fig. 2B) failed to prevent estrogen-induced down-regulation. In Fig. 2B (*left panel*) and in subsequent figures, ER appears as a doublet. The appearance of the doublet is spurious from gel to gel but is only present in estrogen-treated groups. The higher molecular weight form may, therefore, represent an estrogen-dependent posttranslational modification such as phosphorylation. Neither form of ER is preferentially lost in response to estrogen. These data indicate that the mechanism employed to elicit a rapid loss of receptor protein does not require *de novo* protein synthesis or transcription.

ER Protein Is Degraded in Response to Estrogen

The half-life of ER protein is estimated at approximately 3–5 h (16–18). Pulse chase analysis of metabolically labeled ER in whole-cell extracts (Fig. 3) indicates that ER half-life in PR1 cells is greater than 3 h but is within a similar time range as the half-life reported in breast and uterus. This result supports the notion that decreased ER synthesis cannot account for the rapid decline in ER protein steady state levels since presynthesized ER levels do not change significantly within 2 h. In addition, the data imply that estrogen may induce degradation of ER protein. Figure 3 illustrates that the half-life of liganded ER is approximately 1 h. Nonspecific bands that coimmunoprecipitate with ER are not regulated by estrogen and serve as an internal control. This 3-fold change in ER turnover rate is greater than that reported in MCF-7 cells measured by density shift technique and binding of [^{3}H]tamoxifen aziridine (16, 18) and contrasts with previous studies in uterine cells (17, 19). These findings show that estrogen can directly control ER protein by inducing proteolysis and further highlight the importance of receptor protein regulation in lactotrope cells.

Inhibitors of Proteasome-Mediated Proteolysis Prevent ER Turnover

A number of previous studies have addressed potential proteases that may degrade ER (20–23), but no one protease has emerged as a definitive candidate for mediating estrogen-induced down-regulation. The role of three major intracellular proteases (calpain, proteasome, and lysosomal enzymes) was examined by blocking protease activity with a series of inhibitors (Fig. 4A). High concentrations of each inhibitor were used intentionally to ensure that any potential effects might be noticed. The multicatalytic proteasome is responsible for degradation of the majority of cellular proteins. Rock *et al.* (24) demonstrated that proteasome peptidase activity could be blocked by exposure to peptide aldehyde inhibitors including MG115 and

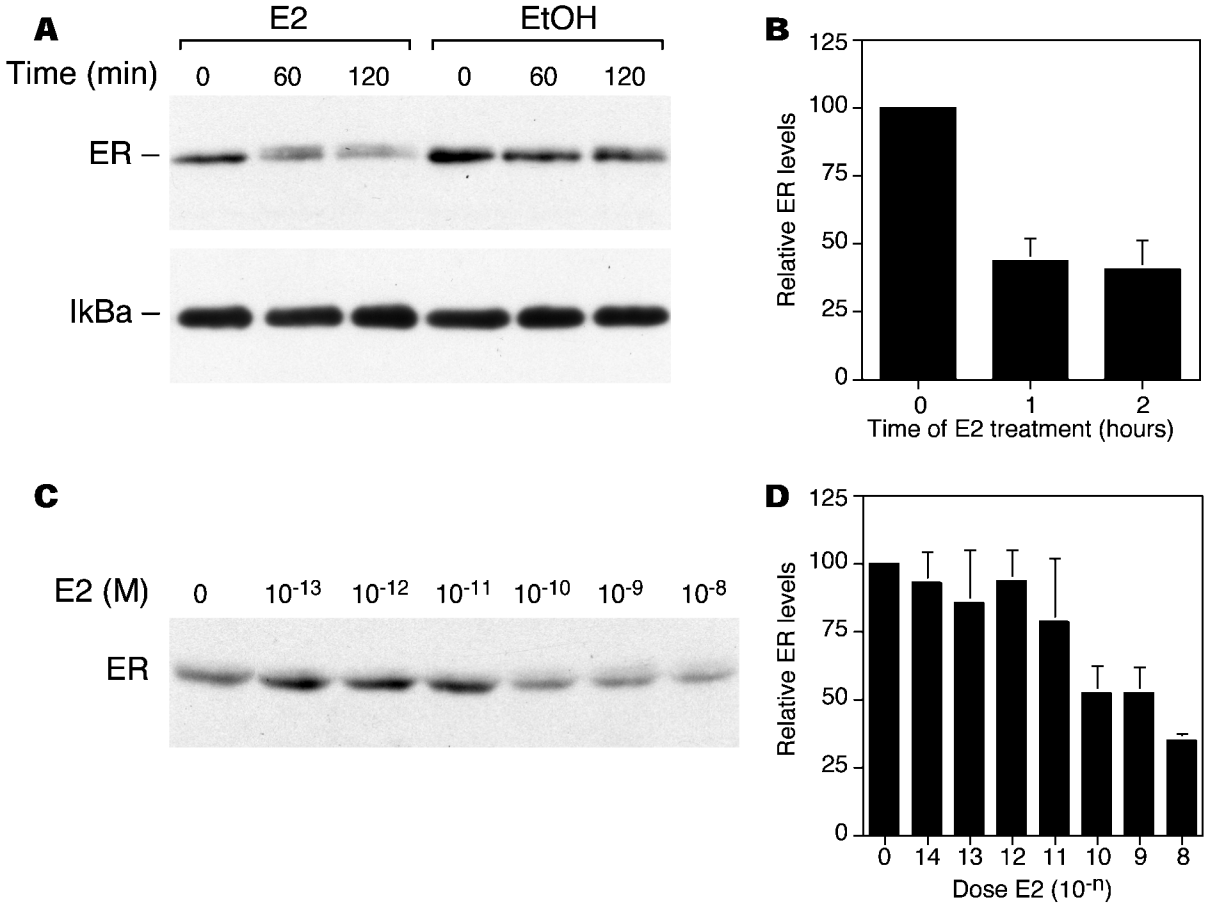

Fig. 1. Effect of Estrogen on Total ER Protein Levels in PR1 Lactotrope Cells

A, Time course of ER response to estrogen. Cells were treated for the indicated length of time with 10 nM 17β-estradiol (E_2). After treatment cells were lysed directly in SDS sample buffer, and proteins were separated by gel electrophoresis in a 7.5% acrylamide gel. Protein was transferred to nylon membrane, and Western analysis was performed with antibody to rat ER. A representative Western blot analysis of ER protein in total cell extract is shown in the *upper panel*. The *lower panel* shows the same Western reprobed with anti-IκBα antibody as a loading control. B, Quantification of time course analysis of ER response was performed by laser densitometry. Cumulative data from four independent experiments are shown. Relative ER levels are represented by the mean ± SEM relative to EtOH-treated controls. Statistical analysis by ANOVA followed by Student's paired *t* test indicate that E_2 treatment results in a significant decrease in ER levels, $P < 0.01$. C, Dose response of estrogen on ER protein levels. Equivalent numbers of PR1 cells were treated for 2 h with varying doses of E_2 as indicated. A representative Western analysis of total ER protein in whole-cell extract is shown. D, Quantification of dose-response analysis was performed by laser densitometry. Data represent the mean ± SEM for three independent experiments relative to untreated controls.

N-acetyleucylleucylnorleucinal (ALLnL). Pretreatment of PR1 cells with MG132, a more potent derivative of MG115, and ALLnL prevented estrogen-induced loss of ER. In contrast, calpeptin and the peptide inhibitor *N*-acetylleucylleycylmethional (ALLM), which preferentially block calpain activity, failed to prevent ER degradation. E64D and NH_4CL, which inhibit cysteine proteases and lysosomal function, respectively, were also without effect. These results suggest that proteasome activity may regulate ER response to estrogen. However, protease inhibitors do not exhibit strict specificity. In the case of the proteasome, the rank order of potency of peptide aldehyde inhibitors directly reflects their specific activity against proteasome function (24). Thus, the relative effectiveness of these inhibitors can be used as a measure of specificity for the proteasome. Dose response experiments were performed with MG132 and ALLnL to determine their relative potencies (Fig. 4B). As a control, similar experiments were conducted with the calpain inhibitor, calpeptin. Examination of data in Table 1 indicates that MG132 was the most effective, preventing ER degradation to a dose of 0.03 μM. ALLnL was less effective but blocked ER degradation between 100 uM and 12.5 μM. L-1-Tosylamido-2-phenylethyl chloromethyl ketone (TPCK), a serine protease inhibitor that weakly inhibits proteasome activity, partially prevented ER proteolysis at a high dose of 12.5 μg/ml (Fig. 4A).

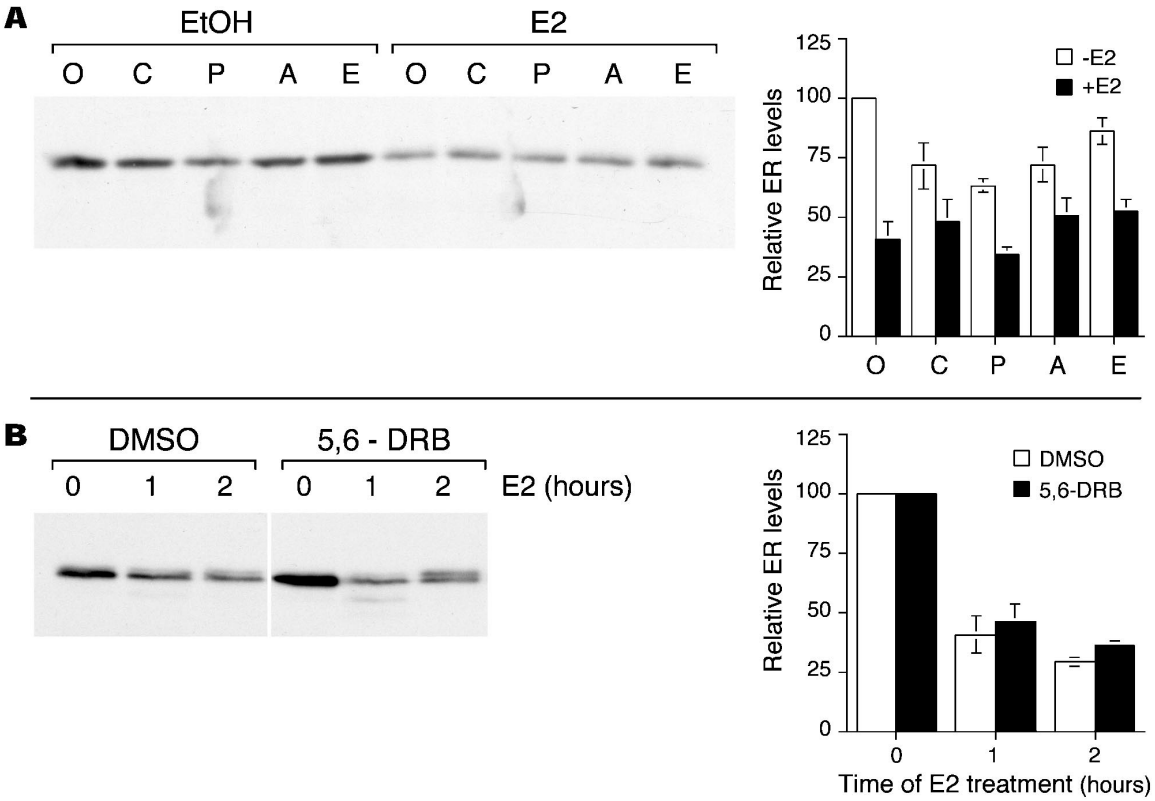

Fig. 2. Inhibition of Protein Synthesis and Transcription Do Not Effect Rapid Turnover of ER Protein

A, PR1 cells (1×10^6) were aliquoted into Optimem media after 3 days in estrogen-free conditions. Protein synthesis was inhibited by a 30-min preincubation with 10 μg/ml of each of the designated inhibitor: cyclohexamide (C), puromycin (P), anisomycin (A), and emetine (E). After preincubation, cells were treated with either ethanol (EtOH) or 10 nM E_2 for 2 h. Total cell extract was obtained by lysing cells directly in sample buffer. The entire sample was analyzed by SDS-PAGE followed by Western analysis with antibody directed against rat ER. Shown is a representative Western blot (*left*), and the quantification of three independent experiments by laser densitometry (*right*) The data represent the results of three independent experiments. B, Cells (1×10^6) were preincubated with 100 μM of transcriptional inhibitor, 5,6-DRB, or DMSO for 30 min before treatment with 10 nM E_2 for 1 and 2 h. ER protein was analyzed as described in panel A. Laser densitometric measurement of total ER levels is shown to the *right* and represents the mean ± SEM relative to controls for three independent experiments.

Calpeptin was ineffective at all doses tested. The order of potency exhibited by these inhibitors in our studies (MG132 > ALLnL) is identical to that previously reported by Rock *et al.* (24). In addition, the weak activity of TPCK against proteasome function is reflected in its partial antagonist action at high doses. These data support the conclusion that estrogen can regulate ER protein directly through a proteasome-mediated pathway. In addition, the ability of the MG132 and ALLnL to increase ER levels relative to controls suggests that the proteasome may also be involved in basal turnover of receptor.

To ensure that proteasome inhibitors were acting directly on ER protein, steady state levels of ER mRNA were examined in the presence and absence of ALLnL and E_2. Figure 5A shows a representative autoradiogram of a Northern blot hybridized with a radiolabeled probe of human ER cDNA. To control for equivalent loading, blots were rehybridized with probe for glyceraldehyde phosphate dehydrogenase (GAPDH). The results of multiple Northern analyses are presented quantitatively in Fig. 5B. Our data show that neither pretreatment with proteasome inhibitor nor treatment with E_2 alters steady state levels of ER mRNA within the first 2 h of treatment. To further verify that the action of the inhibitor was limited to ER protein, pulse chase experiments were performed (Fig. 6). As shown in the more detailed time course of ER half-life (Fig. 3), estrogen induces greater than 50% loss of ER within 2 h. We, therefore, chose the 2-h time point to examine the effect of preventing proteasome function on estrogen-induced shortening of ER half-life. In confirmation of our previous results, estrogen treatment causes a dramatic decrease in ER half-life. Addition of proteasome inhibitor maintained receptor levels comparable to those of controls in both the presence and absence of estrogen. Collectively, these results show that inhibition of proteasome function prevents estrogen-induced proteolysis of ER protein.

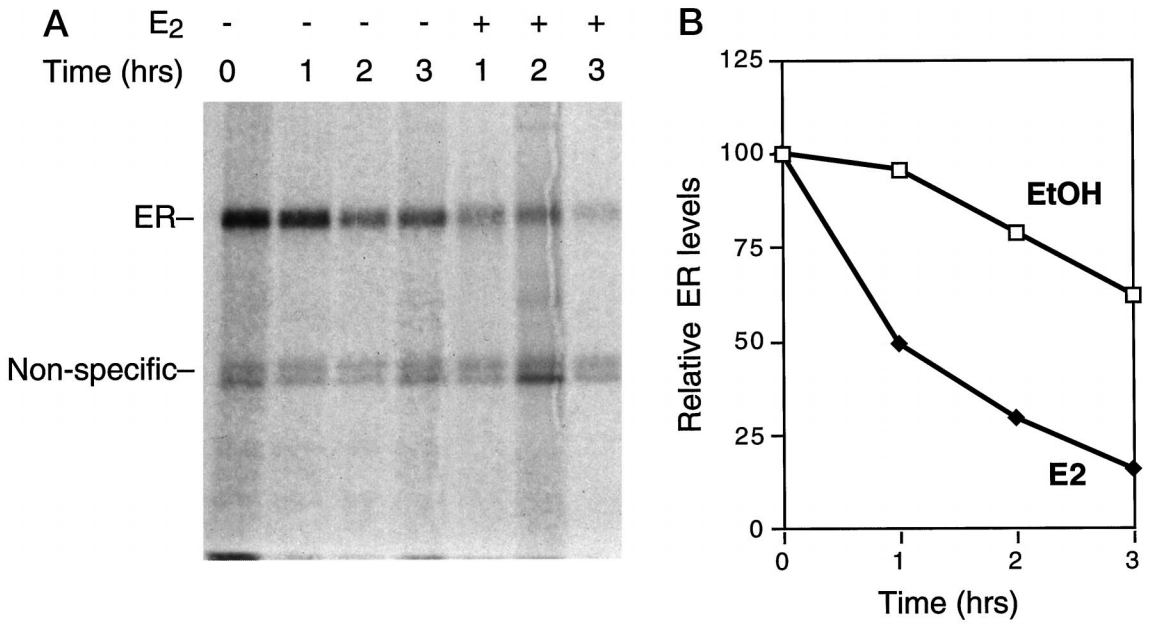

Fig. 3. Estrogen Induces Degradation of ER Protein

Pulse chase analysis was performed as described in *Materials and Methods*. Cells were labeled with ^{35}S-methionine for 2 h and were subsequently chased for the indicated length of time in complete phenol red-free media with 10% stripped serum in the absence (−) or presence (+) of 10 nM E_2. ER was isolated by immunoprecipitation and analyzed by SDS-PAGE. B, ER level experiments were quantified with a PhosphoImager and Imagequant software. Relative ER levels were determined as a percentage of the EtOH-treated group before chase (time 0). Data represent the mean relative ER values of three independent experiments.

Preventing ER Down-Regulation Increases Estrogen-Binding Activity

Examination of our Western and pulse chase analysis suggests that prevention of ER down-regulation with proteasome inhibitors maintains receptor content at a level equivalent to that found in the cells not exposed to estrogen (Figs. 3, 4, and 6). Whole-cell estrogen uptake assays were performed to determine what effect this may have on estrogen-binding activity. For comparison, parallel samples were evaluated by both binding assay and Western analysis. Since binding data requires incubation with [^{3}H]estradiol, there can be no measure of ER in the absence of estrogen. Therefore, ER in unstimulated cells is represented by Western only. In accordance with our previous data, Fig. 7A illustrates that total ER protein content in cells pretreated with proteasome inhibitor is qualitatively similar to those in nontreated cells. This level is higher than those pretreated with solvent since [^{3}H]estradiol induces degradation of receptor protein. Surprisingly, pretreatment with proteasome inhibitor increased specific binding by 1.75-fold relative to those pretreated with dimethylsulfoxide (DMSO) (Fig. 7B). In light of the Western analysis, the binding activity in cells pretreated with ALLnL more closely reflects the receptor content in unstimulated cells. This implies that inclusion of a proteasome inhibitor increases estrogen-binding activity not by increasing receptor number but by maintaining it at the level of nonstimulated cells. These findings suggests that since ER protein is down-regulated during the procedure time required to measure specific binding, the resultant measurement may only account for receptor that remains after rapid proteolysis has taken place. They further suggest that binding measurements in the absence of proteasome inhibitors may underestimate cellular content of ER.

Proteasome-Mediated Proteolysis of ER Is not Unique to Lactotrope Cells

Autologous down-regulation of ER has been examined in a number of systems including uterine cells (17, 19), MCF-7 breast cancer cells (3, 4) (9–12), and cells stably transformed with expression plasmids of mouse (13) and human ER (7). The majority of these studies focus on estrogen's action after several hours, sometimes days, of stimulation. The rapid response to estrogen exhibited by the PR1 cells revealed the role of proteasome-mediated proteolysis in the regulation of ER protein. To determine whether proteolytic regulation of ER is unique to lactotropes, we examined whether ALLnL affected estrogen regulation of ER protein in MCF-7 breast cancer cells, a model system in which down-regulation has been studied extensively. Using an identical treatment regimen, as described for the PR1 lactotropes, the effects of blocking proteolysis on

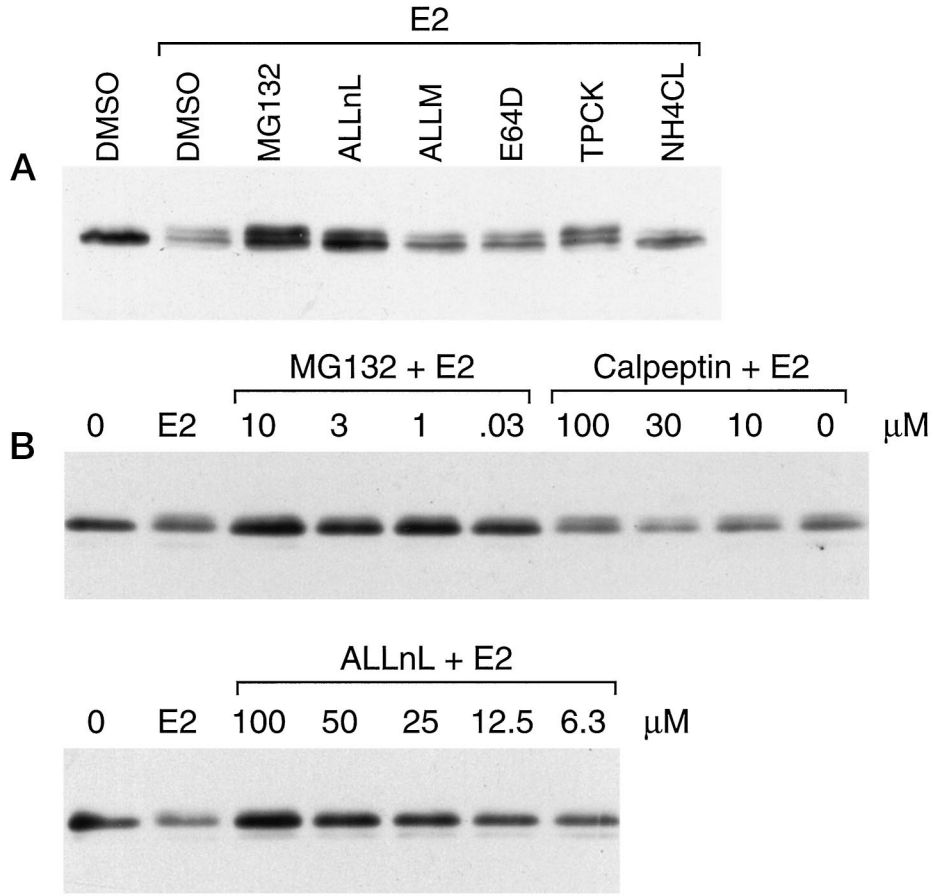

Fig. 4. Proteasome Inhibitors Prevent ER Degradation

A, Equivalent number of PR1 cells ($(1 \times 10^6$/ml) were pretreated with DMSO, MG132 (50 μM), ALLnL (100 μM), ALLM (100 μM), E64D (50 μg/ml), TPCK (12.5 μg/ml), or NH_4Cl (50 mM) for 30 min. Samples were then treated for an additional 2 h with EtOH (lane 1) or 10 nM E_2. After treatment, cells were lysed in SDS sample buffer, and the entire sample was subjected to SDS-PAGE and Western analysis for ER. B, PR1 cells were treated as in panel A with the designated concentrations of MG132 (*upper panel*), Calpeptin (*upper panel*), and ALLnL (*lower panel*). ER levels were visualized after Western analysis of whole-cell lysates.

Table 1. Effectiveness of Inhibitors on Estrogen-Induced ER Down-Regulation

Inhibitor	Dose	Relative ER Level	Inhibitor	Dose	Relative ER Level
None		41.9 ± 5.9	MG132	50 μM	136 ± 26.9
E64D	50 μg/ml	48.1 ± 1.0		10 μM	238 ± 4.9
TPCK	12.5 μg/ml	66.5 ± 10.6		3 μM	213 ± 61.9
NH_4Cl	50 mM	47.5 ± 12.1		1 μM	218.2 ± 18.8
ALLM	100 μM	47 ± 2.0		0.3 μM	152.7 ± 8.7
			ALLnL	100 μM	130 ± 14.6
Calpeptin	100 μM	48.5 ± 13.9		50 μM	126.9 ± 25.7
	30 μM	24.8 ± 3.5		25 μM	117.8 ± 18.0
	10 μM	34.2 ± 20.2		12.5 μM	95.6 ± 18.0
	1 μM	42.3 ± 11.1		6.25 μM	49.6 ± 1.9

Western analyses of inhibitor studies described in Fig. 4 were analyzed by laser densitometry. The groups were pretreated with the designated inhibitor followed by a 2-h treatment with 10 nM E_2. The level of ER was determined relative to controls not treated with E_2. The data represent the mean ± SEM for a minimum of three independent experiments.

ER protein levels in MCF-7 cells was examined. Western analysis shown in Fig. 8 illustrates that pretreatment with ALLnL prevented down-regulation of ER protein in response to estrogen exposure. The demonstration that this mechanism functions in both PR1 cells and MCF-7 cells shows that proteolytic regulation of ER extends beyond rat lactotrope cells to human breast cancer cells and suggests that

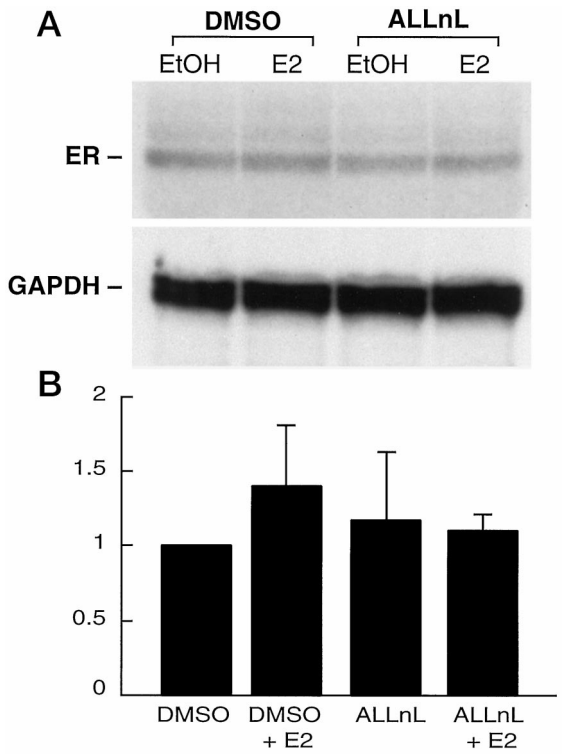

Fig. 5. ER mRNA Levels Do Not Change in Response to E_2 or ALLnL

PR1 cells were pretreated with DMSO or ALLnL (100 μM) for 30 min followed by treatment with either EtOH or E_2 (10 nM). After treatment, 20 μg of total RNA were isolated and separated by electrophoresis in a 1% formaldehyde gel. A, Representative Northern analysis of 20 μg of total RNA hybridized with radiolabeled probe for human ER (*upper panel*) or mouse GAPDH (*lower panel*). B, ER mRNA levels were measured by PhosphoImager analysis of three independent experiments. ER mRNA levels were normalized to GAPDH mRNA levels in the same lane to correct for loading differences. Data are presented relative to the untreated DMSO/EtOH (DMSO) control, which is arbitrarily set at 1.

it may be a common regulatory mechanism used by estrogen to control ER protein.

DISCUSSION

Estrogen induces down-regulation of its receptor as part of a classical endocrine feedback loop. The mechanism of this regulation has been studied previously in a number of model systems. Work conducted in estrogen target cells, such as breast and uterus, and in cells expressing exogenous ER implicates both direct transcriptional repression and posttranscriptional mechanisms (7, 9, 11–13). In line with the transcriptional regulatory role of estrogen, earlier reports focus on changes in ER mRNA levels after several hours and days of stimulation. To our surprise, down-regulation of ER protein in PR1 lactotrope cells occurred rapidly in 1 h. The rate of ER protein loss observed in PR1 cells is faster than previously reported changes in ER mRNA levels in other model systems. Indeed, our results indicate that estrogen induces the loss of ER protein independent of protein synthesis and transcription. This observation prompted us to reexamine the regulatory mechanisms governing autologous down-regulation of ER, not from the perspective of estrogen's action on transcription, but from the perspective of potential actions of estrogen directly on ER protein.

Western analysis of steady state levels showed a rapid loss of total ER protein content in response to estrogen (Fig. 1). We used pulse chase analysis to directly demonstrate that estrogen induces degradation of ER protein and shortens its half-life from greater than 3 h to 1 h. Previous studies that examine the effect of estrogen on ER half-life report conflicting results. For example, while estrogen shortens ER half-life in MCF-7 cells (4, 16), it has no effect in primary uterine cells (17, 19) and lengthens ER half-life in COS cells transfected with mouse ER (25). This variation may simply reflect differences in model systems. It may also, however, reflect differences in methodology. ER levels are commonly determined based on specific estrogen binding. With the exception of studies by Dauvois *et al.* (25), all reported measurements of ER half-life have been based on binding assays. Comparison of estrogen-binding activity and total ER levels detectable by Western blot (Fig. 7) shows a discrepancy between the total number of ERs present in the cell and the number determined by binding assay. Our studies indicate that within the length of time necessary to measure specific estrogen binding (16), a portion of ER protein is degraded. This is supported by early studies of Horwitz and McGuire (6) who demonstrated that the number of ligand-bound sites extracted from the nucleus drops rapidly after 30 min. Consequently, the ER level estimated by binding assays may not take into account ER that is lost during incubation with radiolabeled ligand. The underestimation of ER in the absence of estrogen could account for the lack of measurable difference in ER half-life in the presence of estrogen. The advantage of pulse chase is that [^{35}S]methionine labeling permits the direct examination of a presynthesized pool of ER protein before and after treatment with estrogen, and it does not require binding studies to assess receptor levels.

Throughout these studies, we focused on total ER content by analyzing ER in whole-cell extracts. Although ER is a nuclear protein, when cellular fractions are prepared to distinguish cytoplasmic and nuclear components, ER can be artifactually extracted with cytoplasmic proteins. Estrogen induces undefined changes in the biochemical properties of ER that prevent extraction of ER with hypotonic buffers. This process, referred to as "transformation," can be characterized by an increase in ER in the nuclear fraction upon stimulation with estrogen. It is possible that changes in ER solubility may account for the increased

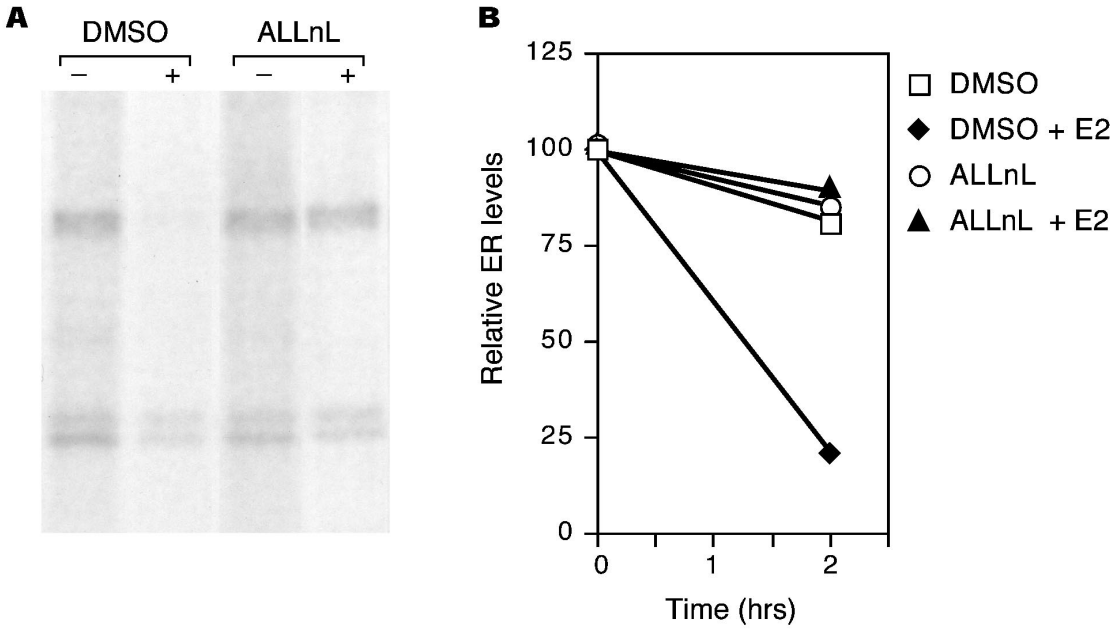

Fig. 6. Blocking Proteasome Function Prevents ER Turnover

PR1 cells were labeled with [^{35}S]methionine for 2 h. After labeling, cells were washed in complete phenol red-free RPMI media with 10% stripped serum and pretreated with DMSO or ALLnL (100 μM) for 30 min (time 0). Cells were subsequently chased for 2 h in complete medium with 10% stripped serum in the absence (−) or presence (+) of 10 nM E_2. Samples were controlled for EtOH content. ER was isolated by immunoprecipitation and analyzed by SDS-PAGE. B, ER levels from three separate pulse chase experiments were quantified by PhosphoImager analysis. Data are presented as a percentage of the DMSO-treated group before the chase ($t = 0$).

half-life of liganded ER reported for mouse ER (25) since the analysis was performed on lysate extracted with high salt. Earlier studies of ER regulation consider "cytoplasmic" and nuclear ER separately. This confounds interpretation of the results in light of the knowledge that ER is exclusively nuclear (26). To simplify our interpretation, we chose to examine whole-cell lysate, which eliminates any changes in ER levels associated with transformation or the extraction process.

We provide evidence that estrogen induces proteolysis of ER via a proteasome-mediated pathway in both the pituitary and MCF-7 breast cancer cell lines. Proteolysis is an important regulatory strategy governing a number of processes, including cell cycle regulation, signal transduction, antigen presentation, and protein quality control. In particular, the proteasome pathway has been implicated as a major protease responsible for the turnover of most proteins in the cell (24). Among those proteins are transcriptional regulators, including p53 (27), MyoD (28, 29), cJun (30), yeast MATα2 (31), and the steroid family corepressor N-CoR (32). In most cases, substrate recognition by the proteasome requires the attachment of multiple ubiquitin moieties to proteins targeted for degradation. Nirmala and Thampan (33) demonstrated that ER is ubiquitinated in an estrogen-dependent manner in normal goat uterus (33). Further, the ubiquitin-activating enzyme, UBA, and ubiquitin-conjugating enzymes, UBCs, can promote *in vitro* degradation of ER protein (34). Consistent with ubiquitin-dependent proteasome function, ubiquitination would serve as a targeting signal to direct ER to the proteasome. Once ER is within the multicatalytic enzyme, it would then be degraded into small peptides, which may account for the lack of observable protein intermediates associated with ER processing (16, 17). This proteolytic mechanism can account for the rapid loss of ER protein that precedes changes in ER RNA.

The threshold nature of this response is illustrated by both dose response and binding studies. Examination of the Western blots shown in Fig. 7A shows that the addition of 200-fold excess of diethylstilbestrol (DES) had no greater effect on ER levels beyond E_2 alone. Dose analysis indicates that significant down-regulation of ER protein does not occur at doses lower than 10^{-10} M E_2. This is a saturable dose of E_2 and is sufficient to occupy 100% of ER in the PR-1 cells (15). Although it remains possible that the detection method is not sensitive enough to measure loss of a small amount of receptor, doses of E_2 sufficient to occupy 50% of the receptors, *i.e.* 10^{-11} M E_2, failed to evoke a corresponding decrease in ER protein. Furthermore, levels of estrogen sufficient to occupy 100% of receptors do not result in the total loss of ER protein. The lack of correlation between receptor occupancy and amount of ER degraded suggests that ligand binding itself does not target ER for degradation.

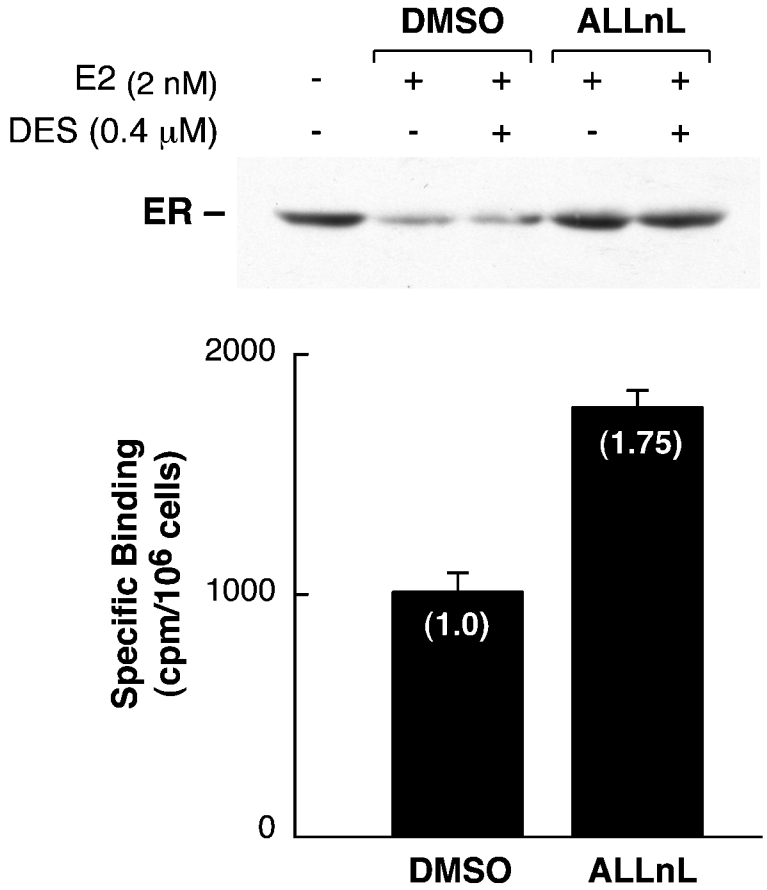

Fig. 7. ALLnL Increases ER-Binding Activity

Whole-cell estrogen uptake assays were performed as described in *Materials and Methods*. PR1 cells (1×10^6/ml) were preincubated with DMSO or ALLnL (100 μM) for 30 min. Cells were then incubated with [^{3}H]estradiol (2 nM) alone or with 200-fold excess of unlabeled DES (0.4 μM) for 2 h. A, Parallel sets of samples were lysed in SDS sample buffer and subjected to Western blot analysis for ER levels. Lane 1 shows control sample that is preincubated with DMSO and treated for 2 h with EtOH. B, After incubation with radiolabeled ligand, cells were washed extensively with PBS/1% BSA and lysed with EtOH. ER-binding activity was determined by scintillation counting of the EtOH lysate. Specific binding was determined by the subtraction of nonspecific (+DES) from total (+E_2 alone). Specific binding data are presented for three independent experiments consisting of duplicate samples for each treatment group. Relative binding activity is shown in *parentheses*.

Since proteasomes are estimated to comprise 1% of the total soluble cellular protein, it is unlikely that they are a limiting component (24). It is possible that downstream events may be required to manifest this response.

Estrogen treatment cannot completely deplete cells of ER protein. Approximately 40% of ER protein remains despite exposure to large doses of estradiol. It is interesting to speculate that certain ER molecules may be resistant to degradation. It is unlikely that the remaining ER represents receptor in a subpopulation of cells since the PR-1 cells do not proliferate significantly (15) and are most likely synchronized when maintained in an estrogen-free environment for 3 days. Moreover, based on molecular weight and epitope recognition, the remaining ER is not ERβ (35) or truncated estrogen receptor products (TERPs) (36), additional ER species reported to be present in pituitary cells. The question remains what distinquishes ER that is destined for degradation from ER that is not. In several cases of proteasome-regulated proteolysis, alterations in the biochemical and physical properties of proteins serve as signals to induce ubiquitination and degradation. The antiestrogen ICI induces rapid degradation of ER (25, 37). In PR1 cells, this appears to operate through a proteasome-mediated mechanism similar to E_2 (E. T. Alarid, unpublished observation). Dauvois *et al.* (20) suggest that ICI disrupts nuclear-cytoplasmic shuttling and promotes cytoplasmic accumulation in certain cells. Interestingly, cytoplasmic ER did not appear to degrade, suggesting that nuclear localization may be a requirement for the degradation process. Regulation of MyoD protein, a skeletal muscle transcription factor, is controlled in part by ubiquitin-proteasome-mediated degradation (28, 29). Abu Hatoum *et al.* (28) recently demonstrated that binding of MyoD to its cognate DNA response element stabilizes MyoD protein and generates a complex that is

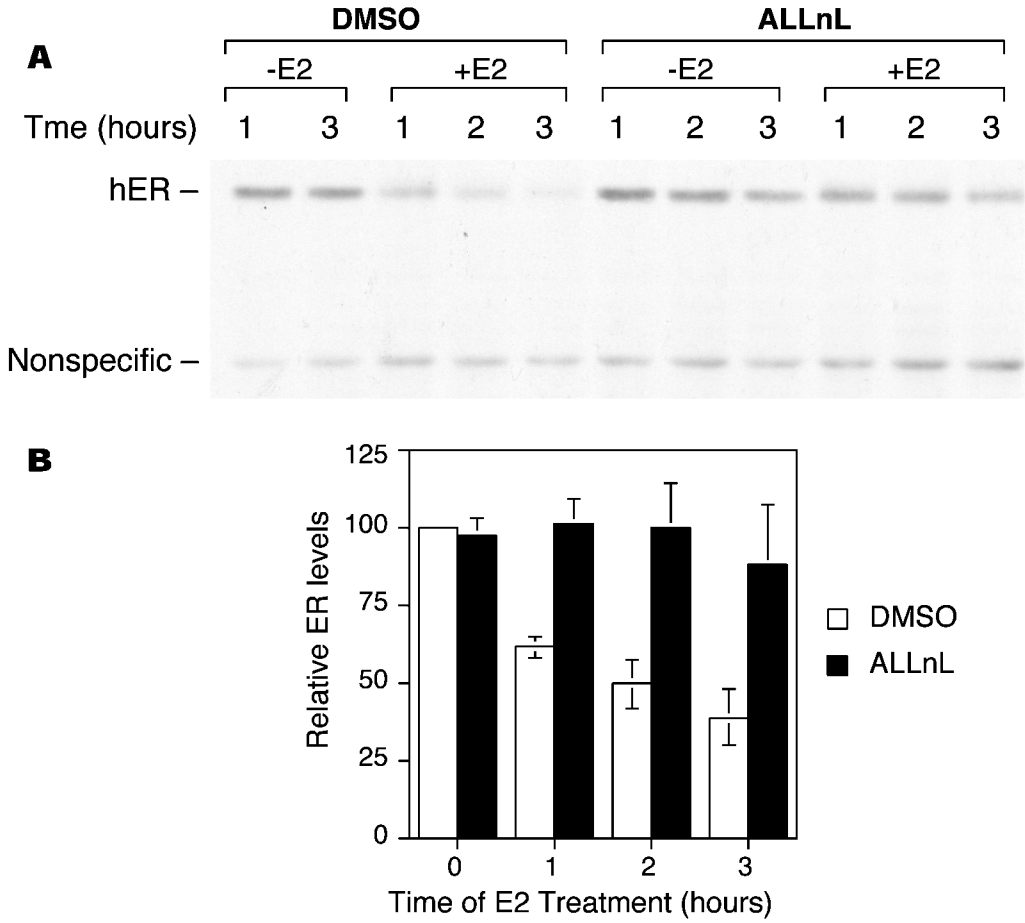

Fig. 8. Proteolytic Regulation of ER in MCF-7 Cells

MCF7 cells (1×10^6/ml) were treated for the indicated length of time with (+E_2) or without (−E2) 10 nM E_2 after 30 min preincubation with DMSO or ALLnL (100 μM). Cells were lysed in SDS sample buffer, and the total sample was subjected to SDS PAGE. Western analysis was performed using antibody (SR1000) directed against an epitope in the hinge region of human ER. A, Representative Western analysis; B, quantification of three independent experiments. The lower molecular weight nonspecific band seen in panel A serves as an internal loading control.

resistant to proteasome degradation. DNA binding may likewise confer resistance to ER protein. Degradation of many proteins is additionally regulated by phosphorylation. Activation of the NF-κB signaling cascade by several extracellular stimuli requires phosphorylation-dependent degradation of IκBα by the ubiqutin proteasome pathway (38). The functional role of phosphorylation of ER is not as yet clearly defined. Specific protein-protein interactions also influence susceptibility to proteasome-mediated degradation. Work by Whitesell and Cook (39) demonstrates that changes in composition of the protein complex associated with GR in the cytoplasm can result in rapid turnover of the GR by the proteasome. Involvement of the coding sequence in down-regulation of ER has been demonstrated previously (7, 13). Further evaluation of the sequence requirement for proteasome-mediated degradation of ER may predicate posttranslational modifications, such as cellular compartmentalization, DNA binding, phosphorylation, or specific protein interactions, that may contribute to ER fate.

We demonstrate that estrogen can regulate ER protein in the absence of transcription and protein synthesis in the pituitary. ER protein regulation can, therefore, be added to the growing number of estrogen actions that do not involve ER-mediated transcription. Predominant among those activities is the activation of signal transduction cascades. Estrogen has been shown to activate MAPK (40) and ERK activity (41) and to lead to the accumulation of second messenger molecules including cAMP (42), inositol phosphate (41, 43, 44), and calcium (45). It has been hypothesized that these nonclassical mechanisms of estrogen action may be mediated through putative membrane-bound receptors that are derived from the same coding transcript (41) and are recognized by the same antibodies as the nuclear ER (41, 46). In the case of ER protein regulation, estrogen's action involves nuclear, not membrane, ERs. Membrane-bound ERs make up

less than 3% of the protein product from nuclear ER transcript (41). Since a significant proportion of ER is down-regulated in response to estrogen, it is most likely that nuclear ER is responsible for mediating estrogen's action. To our knowledge, proteasome-mediated proteolysis of ER is the first identification of a nongenomic mechanism of estrogen action that involves nuclear ERs. Identification of this novel mechanism of estrogen action introduces the possibility for further exploration of nuclear signaling events induced by estrogen that do not involve transcriptional activation.

MATERIALS AND METHODS

Cell Culture

PR1 and MCF-7 cells were grown in high glucose DMEM (Mediatech, Herndon, VA) supplemented with 10% FBS (HyClone Laboratories, Inc., Logan UT), 1 mM sodium pyruvate, 1000 U/ml penicillin, and 1000 mg/ml streptomycin (Life Technologies, Inc., Gaithersburg, MD). Culture conditions were maintained at 10% CO_2 and 37 C in a water-jacketed incubator (Forma Scientific, Inc., Marietta, OH). Cells were passaged using either trypsinization or physical dislodgment with media.

Inhibitor and Estrogen Stimulation

Before treatment, cells were washed with PBS and cultured at 5% CO_2 in phenol red-free and estrogen-free Optimem media (Life Technologies, Inc.) for a minimum of 3 days. While identical results are observed in cells maintained in media with charcoal-stripped serum, we choose to utilize this defined medium to minimize variation that may be associated with the stripping protocol. On the day of treatment, cells were washed with PBS and collected by dispersion with PBS followed by centrifugation. Cell pellets were then resuspended in Optimem that was preequilibrated at 37 C at 5% CO_2 and distributed into 1 ml aliquots of 10^6 cells per tube. In experiments utilizing inhibitors, samples were pretreated with the designated inhibitors for 30 min at 37 C while gently rotating. After pretreatment, cells were exposed to 17β-estradiol (E_2; Sigma Chemical Co., St. Louis, MO) at various doses and for varying lengths of time as indicated in the figure legends. During treatment, the cells were kept at 37 C and rotated continuously. Protease inhibitors tested included ALLnL, ALLM, Calpeptin, TPCK, ethyl(+)-(2S, 3S)-3-[(S)-methyl-1-(3-methylbutylcarbamoyl) butylcarbamoyl]-2-oxiranecarboxylate (E64D), MG132, and NH_4Cl. Controls consisted of pretreatment with DMSO (Sigma Chemical Co.) and treatment with ethanol (EtOH), the solvents for the inhibitors and estradiol, respectively. For practical purposes, ALLnL was used as the preferential proteasome inhibitor. In experiments using protein synthesis inhibitors (cyclohexamide, puromycin, anisomycin, emetine) and transcription inhibitor [5,6-dichloro-1-b-ribofuranosyl benzimidazole (DRB)], cells were pretreated for 30 min as described above before a 2-h treatment with 10 nM E_2. All inhibitors were purchased from Sigma Chemical Co. except MG132 and TPCK, which were gifts from Dr. Shigeki Miyamoto.

Western Blot Analysis

Upon termination of experiments, the cells were pelleted by centrifugation, washed with PBS, and lysed immediately in 2× SDS sample buffer (120 mM Tris-base, 20% glycerol, 2% SDS, 2% β-mercaptoethanol, bromophenol blue, pH 6.8) to yield whole-cell extracts. Whole-cell extracts were boiled and electrophoresed in a 7.5% or 10% SDS-PAGE gel. Proteins were electrophoretically transferred using a Trans-blot Cell (Bio-Rad Laboratories, Inc., Richmond, CA) to nylon membrane (Immobilon-P, Millipore Corp., Bedford, MA) in a Tris-glycine transfer buffer with 20% methanol. The membranes were preblocked in a solution of 5% milk, 0.02% sodium azide, 0.2% Tween 20 in PBS. Membranes were then incubated overnight in the same solution containing primary antibody. The primary antibodies used to detect ER were an anti-ER antibody no. 715 (47) directed against a peptide within the hinge region (amino acids 270–284) of the rat ER and anti-ER antibodies directed against the hinge (amino acids 287–300; SR1000), and C-terminal (amino acids 582–595; SR1010) regions of the human ER (Stressgen, Vancouver, British Columbia, Canada). Antibody to IκBα (C21-Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used to visualize protein not regulated by estrogen and as a loading control. Antibody dilution curves were performed with all primary antibodies to ensure that saturating concentrations were used in the first probe reaction. Blots were washed in PBS containing 0.2% Tween (PBST) before incubation with secondary antibodies conjugated to horseradish peroxidase (HRP) diluted in the identical solution without sodium azide. HRP-conjugated secondary antibodies used were directed against rabbit or mouse IgG (Amersham Pharmacia Biotech, Arlington Heights, IL) as appropriate. After washing in PBST, the signal was visualized using the enhanced chemiluminescence (ECL) detection method (Amersham Pharmacia Biotech) and exposed to x-ray film.

Northern Blot Analysis

Total RNA was isolated from cells using phenol-chloroform extraction as described previously (48). Twenty micrograms of total RNA were electrophoresed in a 1% agarose gel containing formaldehyde and transferred to nylon membrane (Genescreen; NEN Life Science Products, Boston, MA) (49). The RNA was immobilized to the membrane by UV cross-linking (Bio-Rad Laboratories, Inc.). Prehybridization and hybridization of the membranes were performed in a hybridization oven (Robbins Scientific Corp., Sunnyvale, CA) at 55 C in a 25% formamide solution. The blots were probed with ^{32}P-radiolabeled cDNA fragments of the human ER, and mouse GAPDH. Blots were stripped between hybridizations in a boiling solution containing 1% glycerol, 2 mM EDTA, and 0.5% SDS for a minimum of 10 min. Signal was quantified with a PhosphoImager using Imagequant software (Molecular Dynamics, Inc., Sunnyvale, CA). Expression level of ER mRNA was determined by normalizing values to those of the loading control, GAPDH. Values obtained for the untreated DMSO control were set at 1. The data are presented as the mean ± SD of the ER mRNA level relative to the DMSO control for three independent experiments.

Whole-Cell Estrogen Uptake Assay

PR-1 cells that were maintained in Optimem for a minimum of 3 days were aliquoted into microcentrifuge tubes at a concentration of 2×10^6/ml in fresh medium that was preequilibrated to 37 C at 5% CO_2. The cells were pretreated for 30 min with either DMSO or ALLnL at 37 C while rotating. After pretreatment, 2 nM [^{3}H]estradiol (New England Nuclear/Dupont, Boston, MA)was added to all samples. To account for nonspecific binding, 0.4 μM DES (Sigma Chemical Co.) was added in addition to 2 nM [^{3}H]estradiol in a parallel set of samples. During the 2-h treatment period, cells were kept at 37 C and were rotated continuously. All samples were controlled for equivalent amounts of ethanol. Cells were harvested by centrifugation and were washed two times with 1%

BSA in PBS at 4 C. The final pellet was resuspended in ethanol and counted in a scintillation counter. Specific binding was calculated by the subtraction of nonspecific from total binding. Samples within individual experiments were performed in duplicate. The data are presented as the mean + SE of three independent experiments. To compare total ER protein content to ER binding, whole-cell extract from a parallel set of samples was examined by Western blot analysis as described above.

Pulse Chase

Estrogen-deprived PR1 cells were rinsed twice in RPMI media lacking phenol red, methionine, and cysteine (RPMI$^-$, Life Technologies, Inc.). Cells were incubated for 45 min in RPMI$^-$ supplemented with L-glutamine, sodium pyruvate, nonessential amino acids, and 5% stripped serum (50) that had been dialyzed overnight against 0.9% NaCl. Metabolic labeling with [^{35}S]methionine was conducted for 2 h at a concentration of 1 mCi/10^7 cells. After labeling, cells were washed with complete phenol red-free RPMI media containing 10% stripped serum. ALLnL and E_2 treatment was performed as described above for 1, 2, or 3 h in complete phenol red-free RPMI medium containing 10% stripped serum. Treated cells were lysed in a solution consisting of 10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 0.4% NP40, and ER was immunoprecipitated using antirat ER antibody and protein A sepharose (Pharmacia Biotech, Piscataway, NJ). Immunoprecipitate was analyzed by SDS-PAGE. ^{35}S-labeled ER was visualized by autoradiography, and relative values of ER protein were determined with a PhosphoImager using Imagequant software (Molecular Dynamics, Inc.). Data are presented as a percentage of the DMSO control group before exposure to E_2. Values represent three independent experiments.

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Address requests for reprints to: Elaine T. Alarid, Ph.D., Department of Physiology, 120 Service Memorial Institute, 1300 University Avenue, Madison, Wisconsin 53706. e-mail: alarid@physiology.wisc.edu.

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