E6AP and Calmodulin Reciprocally Regulate Estrogen Receptor Stability*

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Estrogen promotes the proliferation of human breast epithelial cells by interacting with the estrogen receptor (ER). Physiological responses of cells to estrogen are regulated in part by degradation of the ER. Previous studies revealed that calmodulin binds directly to the ER, thereby enhancing its stability. Consistent with these findings, cell-permeable calmodulin antagonists dramatically reduced the number of ER in MCF-7 human breast epithelial cells. Here we investigated the molecular mechanism by which calmodulin attenuates ER degradation. MG132 and lactacystin, inhibitors of the ubiquitin-proteasome pathway, prevented the calmodulin antagonist CGS9343B from reducing the amount of ER in MCF-7 cells. In contrast, protease inhibitors afforded no protection. Moreover, CGS9343B enhanced ER ubiquitination. A point mutant ER construct that is unable to bind calmodulin, termed ER ΔCaM, is ubiquitinated to a greater extent than wild type ER. The ubiquitin-protein isopeptide ligase E6-associated protein (E6AP) associated with and promoted the degradation of ER. The possible convergence of calmodulin and E6AP on ER degradation was examined. ER ΔCaM bound E6AP with higher affinity than that of wild type ER. Moreover, calmodulin attenuated the interaction between ER and E6AP in a Ca2+-dependent manner. Collectively, our data reveal that E6AP is a component of ER degradation via the ubiquitin-proteasome pathway and that Ca2+/calmodulin modulates this degradation mechanism. These results have potential implications for the development of selectively targeted therapeutic agents for breast cancer.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture reagents were purchased from Invitrogen, and fetal bovine serum (FBS) was obtained from BioWhittaker. Charcoal-treated FBS was from Cocalico Biologicals Inc. MCF-7 and T47D breast epithelial cells and COS-7 green monkey kidney cells were obtained from American Type Culture Collection. CGS9343B was generously donated by Drs. E. Moret and B. Schmid (Novartis, Switzerland). Lactacystin, MG132, calpeptin, and the calpain inhibitor II N-acetyl-leucine-leucine-methioninal (ALLM) were purchased from Calbiochem. Protein A- and protein G-Sepharose were from Amersham Biosciences. FuGENE 6 was obtained from Roche Applied Science. Polyvinylidene difluoride (PVDF) membrane was purchased from Millipore Corp. Nonimmune rabbit serum (NIRS), 17β-estradiol (E2), and trifluoperazine (TFP) were from Sigma. Purified human ER and pig brain calmodulin were purchased from Panvera and Ocean Biologics, respectively.
Antibodies—Anti-ER monoclonal (Ab-15) and polyclonal antibodies were from Neomarkers and Santa Cruz Biochemistry, respectively. Anti-His and anti-HA (12CA5) antibodies were from Roche Applied Science. Anti-Myc antibody was from Maine Biotechnology Inc. The anti-E6AP antibody has been described previously (17).

Plasmids—The plasmids for wild type and mutant E6AP have been described previously (17, 18). The mutant E6AP (named E6AP C833A) has a cysteine to alanine (Cys → Ala) mutation at amino acid 833, which eliminates the catalytic activity of the protein. The Nedd4 mutant (named Nedd4 C744A) was generated by a Cys → Ala at amino acid 744 (19) of pcDNA-myc-Nedd4, which similarly eliminated catalytic activity.

pGEX-2T-E6AP (20) and pMT107 (21) were described previously.

A deletion mutant and a point mutant ER unable to bind calmodulin were developed. These constructs are described in detail elsewhere (22). Briefly, ERΔ298–317 was generated by deleting amino acid residues 298–317 from pcDNA3-myc-ER by using PCR. Site-directed mutagenesis of pcDNA3-myc-ER was performed with the QuickChange site-directed mutagenesis kit (Stratagene). The mutant cDNA was amplified by PCR with Pfu turbo DNA polymerase using the oligonucleotides 5'-CCAGGCCCAGTCAAGCAAGCCTTACAGAG-3' (mutated residues are underlined). These changes result in replacement of Ile-298 with Glu and lys-299 with Asp. The construct is termed ERΔCaM. The sequence of both constructs was confirmed by DNA sequencing.

Cell Culture and Transfection—MCF-7 and COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) FBS. T47D cells were grown in RPMI 1640 medium supplemented with 10% (v/v) FBS. MCF-7 and COS-7 cells were main-}

In Vivo Ubiquitination—COS-7 cells were cotransfected with pMPT107 (His6-tagged ubiquitin) and pcDNA3-ER or pcDNA3-ERαCaM. Forty eight hours post-transfection, cells were treated for 2 h with 10 μM MG132 or vehicle, followed by further incubation with 40 μM CMS9343B or an equal volume of vehicle (ethanol) for 16 h. Lysates were harvested in 1 ml of buffer C (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.1% protease inhibitor mixture, and 1 mM PMSF). After preclearing with 20 μl each of protein G- and protein A-Sepharose beads, equal amounts of protein lysate were immunoprecipitated with anti-His or anti-ER antibodies for 4 h at 4°C. Immune complexes were collected and washed five times with buffer C, and the samples were processed by Western blotting.

In Vitro Ubiquitination—GST-E6AP, E1-His (a kind gift from Jeff Parvin), and UbcH5c-His (a kind gift from Richard Baer) were expressed in Escherichia coli BL21 (DE3). GST-E6AP was purified by gluthathione-Sepharose chromatography. E1-His and UbcH5c-His were purified by nickel affinity chromatography using Ni²⁺-nitrilotriacetic acid-agarose.

RESULTS

Calmodulin Antagonists Reduced the Amount of ER Protein in Breast Epithelial Cells—We have documented previously (11) that incubation of MCF-7 cells with calmodulin antagonists significantly reduced the amount of ERα protein. This reduction was due to enhanced degrada-
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FIGURE 1. Calmodulin antagonists reduced endogenous ER—A, T47D breast epithelial cells were incubated with vehicle (−) or 40 μM CGS9343B, or 10 μM TFP (+) for 16 h in the absence (E0H) or the presence of 10 nM E2. After lysis, equal amounts of protein were resolved by SDS-PAGE and transferred to PVDF, and the membranes were probed for ER. Data are representative of three experimental determinations. B, COS-7 cells, transiently transfected with wild type ER (WT), ΔA298–317, or ERΔCaM (Ile-298 replaced with Glu and Lys-299 changed to Asp) were incubated with vehicle (−) or 40 μM CGS9343B (+) for 16 h. Equal amounts of protein lysate were processed by Western blotting for ER. Data are representative of two independent experimental determinations.

Effect of Proteasome and Protease Inhibitors on ER Degradation—The data in Fig. 2 suggested that calmodulin may inhibit ER ubiquitination. To test this hypothesis, COS-7 cells were cotransfected with ER and His-tagged ubiquitin, and the extent of ER ubiquitination was examined by probing anti-His immunoprecipitates for ER. Ubiquitin is conjugated to multiple lysine residues of target proteins and forms polyubiquitin chains (27). Therefore, ubiquitin-tagged proteins can sometimes be seen as a ladder of higher molecular weight species on SDS–PAGE (6). Incubation of cells with MG132 enhanced ER ubiquitination (Fig. 3; ubiquitinated forms of ER are visible as a smear of higher molecular weight bands). Similarly, incubation with CGS9343B alone slightly increased ER ubiquitination but to a lesser extent than MG132 (Fig. 3A). When cells were treated with both MG132 and CGS9343B, ER ubiquitination was further enhanced. Essentially identical results were obtained when ubiquitinated proteins were isolated by immunoprecipitation with anti-ER antibodies and identified by probing blots for His-ubiquitin (data not shown). These observations suggest that CGS9343B promotes ER degradation by enhancing ER ubiquitination.

Ubiquitination of Wild Type ER and ERΔCaM—The role of calmodulin in ER ubiquitination was explored further with ERΔCaM, a point mutant ER construct that cannot bind calmodulin (22). COS-7 cells were transiently transfected with wild type ER or ERΔCaM, and the extent of ubiquitination of the two ER proteins was compared. When cells were incubated with MG132, ubiquitination of ERΔCaM was substantially greater than that of wild type ER (Fig. 3B). The expression levels of wild type ER and ERΔCaM were comparable as shown in the Western blot of cellular lysates (Fig. 3B, lower panel).

ER is a Substrate of E6AP—Ubiquitination is a three-step process in which ubiquitin is activated by a ubiquitin-activating enzyme (E1), transferred to a ubiquitin-conjugating enzyme (E2) and then transferred to the target by a ubiquitin–protein ligase (E3) (26, 28). E6AP was initially identified as a ubiquitin ligase that cooperates with the human papillomavirus (HPV) E6 oncoprotein to degrade the tumor suppressor p53 (18). A few years ago, the human progesterone receptor was shown to directly interact with E6AP (29). Therefore, we wondered whether E6AP participates in regulation of ER. Initial analysis was to determine whether the E3 ligase activity of E6AP is capable of degrading endogenous ER.Transient transfection of MCF-7 cells with wild type E6AP decreased endogenous ER levels by 21 ± 3.3% (mean ± S.E., n = 3; p < 0.05) (Fig. 4). Further analysis was performed with a catalytically inactive mutant form of E6AP (termed E6AP C833A), which has a cysteine produced by incubating cells with CGS9343B (Fig. 2B). Collectively, these data imply that calmodulin protects the ER from degradation in the ubiquitin-proteasome pathway.
to alanine substitution at amino acid 833 in the active site, rendering it incapable of forming a thiol ester with ubiquitin (17). In contrast to the wild type enzyme, E6AP C833A did not significantly reduce the amount of ER in the cells (Fig. 4). Note that the expression levels of E6AP and C833A were comparable. These data reveal that the E3 ligase activity of E6AP contributes to degradation of ER.

E6AP Associates Specifically with ER—The possible interaction of ER with E6AP was examined by two approaches, namely immunoprecipitation and pull-down with GST fusion proteins. In the first approach, endogenous E6AP was immunoprecipitated from COS-7 cells expressing ER. E6AP coimmunoprecipitated with E6AP (Fig. 5A). Specificity was confirmed by the absence of ER from samples precipitated in parallel with NIRS. Analogous experiments were conducted with MCF-7 cells from which endogenous ER was immunoprecipitated. This analysis revealed that both wild type and mutant E6AP coimmunoprecipitated with ER (Fig. 5B). Although the expression levels of wild type and mutant E6AP were equivalent (see lysate in Fig. 5B), the amount of E6AP C833A that coimmunoprecipitated with ER was substantially more than the amount of wild type E6AP. Similar observations were made in COS-7 cells, which were cotransfected with ER and HA-tagged E6AP C833A. Cells were lysed 24 h later, and equal amounts of protein lysate were resolved by SDS-PAGE and transferred to PVDF. The blot was probed with antibodies to HA (to identify the E6AP) and ER. The amount of ER was quantified by densitometry. Data, expressed relative to the amount of ER in cells transfected with vector, represent the means ± S.E. (n = 3). NS, not significant.

Effect of Calmodulin on the Binding of ER to E6AP—In the second approach, GST–E6AP was incubated with lysates derived from COS cells transfected with ER. GST–E6AP bound ER in COS cell lysates (Fig. 6A). Binding was specific as no ER was detected in lysates incubated

![Image](50x289 to 300x732)

**FIGURE 3.** Calmodulin modulates ER ubiquitination. A, COS-7 cells were transiently cotransfected with His-ubiquitin and either empty pcDNA3 vector (−) or pcDNA3-ER (+). Forty eight hours later, cells were treated with vehicle (−) or 10 μM MG132 for 2 h, followed by incubation with or without 40 μM CGS9343B for 16 h. Equal amounts of protein lysate were immunoprecipitated (IP) with anti-His antibody. Immunoprecipitates were resolved by SDS-PAGE and transferred to PVDF. Immunoblotting (IB) for ER was performed to identify ubiquitinated ER (ER-ub, higher molecular weight multiple bands). Molecular weight markers are depicted on the left. Data are representative of seven independent experimental determinations. B, COS-7 cells were transiently cotransfected with His-ubiquitin and either empty pcDNA3 vector (V), pcDNA3-ER (ER WT), or pcDNA3-ER&CaM (ΔCaM). Forty eight hours later, cells were treated with vehicle (Me2SO (DMSO)) or 10 μM MG132 for 16 h. Equal amounts of protein lysate were processed by SDS-PAGE and Western blotting for ER (Lysate, lower panel). In addition, equal amounts of protein lysate were immunoprecipitated (IP) with anti-ER antibodies. Immunoprecipitates were resolved by SDS-PAGE and transferred to PVDF. The blot was probed (IB) for His (upper panel) to identify ubiquitinated ER (ER-ub, higher molecular weight multiple bands). Molecular weight markers are depicted on the left. Data are representative of three independent experimental determinations.

![Image](314x508 to 564x732)

**FIGURE 4.** Effect of E6AP on ER levels in MCF-7 cells. A, MCF-7 cells were transiently transfected with pcMV4 vector (V), HA-tagged wild type E6AP, or HA-tagged E6AP C833A (C833A). Cells were lysed 24 h later, and equal amounts of protein lysate were resolved by SDS-PAGE and transferred to PVDF. The blot was probed with antibodies to HA (to identify the E6AP) and ER. The amount of ER was quantified by densitometry. Data, expressed relative to the amount of ER in cells transfected with vector, represent the means ± S.E. (n = 3). NS, not significant.

**TABLE 1.** Summary of effects of E6AP on ER levels in MCF-7 cells. Values are means ± S.E. (n = 3). NS, not significant.

| Treatment | Effect of E6AP on ER levels |
|-----------|-----------------------------|
| Vehicle | −0.1 ± 0.05 | −NS |
| HA-E6AP | −0.1 ± 0.05 | −NS |
| V + E6AP | −0.1 ± 0.05 | −NS |
| V + C833A | −0.1 ± 0.05 | −NS |

**TABLE 2.** Summary of effects of E6AP on ER levels in COS-7 cells. Values are means ± S.E. (n = 3). NS, not significant.

| Treatment | Effect of E6AP on ER levels |
|-----------|-----------------------------|
| Vehicle | −0.1 ± 0.05 | −NS |
| HA-E6AP | −0.1 ± 0.05 | −NS |
| V + E6AP | −0.1 ± 0.05 | −NS |
| V + C833A | −0.1 ± 0.05 | −NS |

**TABLE 3.** Summary of effects of E6AP on ER levels in MCF-7 cells. Values are means ± S.E. (n = 3). NS, not significant.

| Treatment | Effect of E6AP on ER levels |
|-----------|-----------------------------|
| Vehicle | −0.1 ± 0.05 | −NS |
| HA-E6AP | −0.1 ± 0.05 | −NS |
| V + E6AP | −0.1 ± 0.05 | −NS |
| V + C833A | −0.1 ± 0.05 | −NS |

**TABLE 4.** Summary of effects of E6AP on ER levels in COS-7 cells. Values are means ± S.E. (n = 3). NS, not significant.

| Treatment | Effect of E6AP on ER levels |
|-----------|-----------------------------|
| Vehicle | −0.1 ± 0.05 | −NS |
| HA-E6AP | −0.1 ± 0.05 | −NS |
| V + E6AP | −0.1 ± 0.05 | −NS |
| V + C833A | −0.1 ± 0.05 | −NS |
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FIGURE 5. E6AP associates specifically with ER. A, COS-7 cells were transfected with pcDNA3-ER, followed 36 h later by the addition of 10 μM MG132. After incubating for 12 h, cells were lysed, and equal amounts of protein were subjected to SDS-PAGE and Western blotting (Lysate). In addition, equal amounts of protein lysate were immunoprecipitated (IP) with anti-E6AP antibodies or nonimmune rabbit serum (NIRS), resolved by SDS-PAGE, and transferred to PVDF membrane. Western blots were probed with anti-E6AP and anti-ER antibodies. B, MCF-7 cells were transiently transfected with pcMV4 vector (V), pcMV4-HA-E6AP (E6AP), or pcMV4-HA-E6AP-C833A (C833A). Cells were lysed 24 h later, and equal amounts of protein lysate were immunoprecipitated (IP) with anti-ER antibodies. Western blots were probed with anti-E6AP and anti-ER antibodies. C, COS-7 cells were cotransfected with pcDNA3-ER and either pcMV4 vector, E6AP, or E6AP C833A. After 48 h, cells were lysed and processed as described for B. D, COS-7 cells were cotransfected with pcDNA3-ER and catalytically inactive (C744A) or wild type (WT) pcDNA-Myc-Nedd4. Equal amounts of protein lysate were resolved by Western blotting (lysate). In addition, equal amounts of protein lysate were immunoprecipitated (IP) with anti-ER antibody or NIRS. Western blots were probed with anti-Myc (to detect the Myc-Nedd4) and anti-ER antibodies. All data are representative of at least three independent experimental determinations.

with GST alone. The enhanced ubiquitination of ER in the absence of calmodulin binding, coupled with the association between E6AP and ER, led to the hypothesis that calmodulin may modulate the ER-E6AP interaction. Three interrelated experimental methods were used to test this hypothesis. In the first strategy, the binding of ERΔCaM to GST-E6AP was compared with that of wild type ER. Binding of ERΔCaM to E6AP was substantially greater than that of wild type ER (Fig. 6A).

The second strategy was to perform direct in vitro competition assays to assess ER binding to E6AP in the presence of calmodulin. Pure ER bound to GST-E6AP (Fig. 6B). When ER was preincubated with calmodulin in the presence of Ca2+, the binding of ER to GST-E6AP was substantially reduced (Fig. 6B). In contrast, when Ca2+ was chelated with EGTA, calmodulin did not attenuate ER binding to GST-E6AP (data not shown). These findings are consistent with our prior observations that calmodulin binds ER in Ca2+-dependent manner (11).

The effect of the cell-permeable calmodulin antagonist CGS9343B was the third strategy. Incubating MCF-7 cells transfected with E6AP with CGS9343B increased by 2.5-fold the amount of wild type E6AP that coimmunoprecipitated with endogenous ER (Fig. 7). CGS9343B also increased the interaction of E6AP C833A with ER (Fig. 7), although the magnitude of enhancement was much less than that seen with wild type E6AP. Because CGS9343B decreases the binding of calmodulin to ER (11), these results suggest a model in which calmodulin and E6AP compete for ER binding. Disrupting the association of calmodulin with ER facilitates the ER-E6AP interaction.

Calmodulin Reduced ER Ubiquitination in Vitro—An in vitro ubiquitination assay was used to determine whether E6AP directly ubiquитinates ER. We were able to reconstitute ER ubiquitination using pure proteins. In the presence of E1, U2 Ubch5c, and ubiquitin, E6AP catalyzed the ubiquitination of ER (Fig. 8). No ubiquitinated ER was detected when E6AP or ubiquitin was omitted from the assay. Precipitation of ER with Ca2+/calmodulin markedly reduced ubiquitination of ER by E6AP (Fig. 8). Calmodulin had no effect on E6AP-mediated ubiquitination of ER when Ca2+ was chelated with EGTA (data not shown). These data reveal that E6AP is directly responsible for ER ubiquitination and that calmodulin attenuates the reaction.

DISCUSSION

Several lines of evidence couple Ca2+ and calmodulin to breast carcinoma and ER function. These include the following. (i) Calmodulin concentrations are increased in malignant human mammary tissue (31).
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Calmodulin is required for the normal transcriptional function of the ER (22). Together these findings suggest that Ca\(^{2+}\)/calmodulin may participate in ER-induced transcriptional activation and the mitogenic effects of estrogen. An intriguing publication revealed that E\(_{2}\) administration to ovariectomized mice up-regulated, independently of ER, the expression of calmodulin mRNA in the uterus by 4.5-fold (34). These data reveal a reciprocal regulation between calmodulin and estrogen.

An additional biological role of calmodulin in estrogen function is to modulate ER degradation (11). The binding of calmodulin to ER enhanced the stability of ER both in vitro and in intact cells (11). The present study was undertaken to elucidate the molecular mechanism by which calmodulin mediates this effect. Two complementary strategies were adopted. These were the use of a selective, cell-permeable calmodulin antagonist CGS9343B (35) and a point mutant ER (termed ER\(\Delta\)CaM) that is unable to bind calmodulin (22). We developed ER\(\Delta\)CaM by selectively mutating two amino acids (Ile-298 and Lys-299) in the calmodulin-binding domain of ER, thereby abrogating calmodulin binding. CGS9343B enhanced proteolysis of wild type ER but had no effect on the level of ER\(\Delta\)CaM in cells. These data verify that the calmodulin antagonist CGS9343B promotes ER degradation by disrupting the interaction between calmodulin and ER.

The ubiquitin-proteasome pathway is responsible for E\(_{2}\)-dependent down-regulation of ER levels (6–9). We observed that the proteasome inhibitors lactacyclin and MG132 blocked the degradation of ER produced by CGS9343B. Despite a previous report that ER is a substrate of calpain (36), inhibition of calpain activity did not attenuate the enhanced ER proteolysis that occurred in cells incubated with CGS9343B. These results imply that calmodulin stabilizes ER by decreasing its degradation in the ubiquitin-proteasome pathway. This hypothesis is supported by the finding that disruption of the interaction between ER and calmodulin augments ER ubiquitination. Two distinct, complementary strategies yielded essentially identical results. Incubation of MCF-7 cells with CGS9343B, which blocks the association of calmodulin with ER, enhanced ubiquitination of ER. Similarly, ER\(\Delta\)CaM was ubiquitinated to a greater extent than wild type ER.

Ubiquitination of protein substrates is a multistep process that involves the concerted action of at least three classes of enzymes as follows: E1, E2, and E3 (26, 37). E1 activates ubiquitin, a highly conserved 76-residue polypeptide, in an ATP-dependent reaction to generate a high energy thiol ester intermediate. One of at least 25 E2 enzymes specifically binds to one of a group of E3 ligases. E3s catalyze the attachment of ubiquitin to the substrate, the final step in the pathway. The sequential addition of activated ubiquitin moieties onto a lysine residue of the previously conjugated ubiquitin molecule produces a polyubiquitin chain (26). This chain is recognized by the 26 S proteasome, resulting in degradation of the ubiquitinated protein. The E3 ubiquitin ligases are key components that provide selectivity to the pathway by interacting directly with different substrates. E6AP is the prototype of a family of E3 ligases called HECT proteins, all of which contain a C terminus (38). This domain catalyzes ubiquitin transfer, although substrate specificity is provided by other domains. The first documentation of a role for E6AP in nuclear hormone function was by O’Malley and co-workers (29) who described a hormone-dependent regulation of transcriptional activity of the progesterone receptor by E6AP. This effect, which was independent of the ligase function of E6AP, was also noted for glucocorticoid receptor and ER (29). However, neither the interaction of E6AP with ER nor its role in ER ubiquitination was examined in that study.

Published work from several laboratories over the last 5 years has

(ii) Ca\(^{2+}\)/calmodulin stimulates E\(_{2}\) binding to the ER (32). (iii) Calmodulin binds to the ER in a Ca\(^{2+}\)-dependent manner (11, 15, 33), and this increases the \(K_{d}\) value of estradiol binding (14). (iv) Ca\(^{2+}\)/calmodulin stimulates tyrosine phosphorylation and activation of the ER (32). (v) Association of calmodulin with the activated estrogen-ER complex increases the interaction of the complex with ERE (14). (vi) Calmodulin is required for the formation of the ER-ERE complex and for activation of an estrogen-responsive promoter (15). (vii) Calmodulin antagonists prevent E\(_{2}\) from stimulating ER transcription (16, 24). (viii) Binding to

**FIGURE 7.** CGS9343B enhanced the interaction of ER with E6AP. A, MCF-7 cells were transiently transfected with pCMV4-HA-E6AP (E6AP) or pCMV4-HA-E6AP-C883A (C883A). Twenty-four hours later, cells were treated for 2 h with vehicle (−) or CGS9343B (+). Equal amounts of protein lysate were processed by SDS-PAGE, followed by Western blotting with anti-HA and anti-ER antibodies (bottom panel). In addition, equal amounts of protein lysate were immunoprecipitated (IP) with anti-ER antibodies. Immunoprecipitates were resolved by SDS-PAGE and transferred to PVDF. The blot was probed for HA (to identify E6AP) (top panel) and for ER (middle panel). B, the amount of E6AP that coimmunoprecipitated with ER was quantified by densitometry. Data, expressed relative to the amount of E6AP in vehicle-treated cells transfected with wild type E6AP, represent the means ± S.D. (n = 2).

**FIGURE 8.** Calmodulin inhibits E6AP-mediated ubiquitination of ER in vitro. Purified ER was preincubated in vitro without (−) or with (+) calmodulin (CaM). After 10 min, E1 and E2 were added in the absence or presence of 10 μg biotin-tagged ubiquitin and 1 μg of E6AP, and the reaction was allowed to proceed for 30 min. The assay was stopped by adding SDS-PAGE solubilization buffer, and samples were processed by Western blotting. Ubiquitinated protein products were visualized with horseradish peroxidase-conjugated streptavidin. Ubiquitinated ER (ER-ub) is labeled. The identity of the ubiquitinated protein ~50 kDa is unknown. Molecular weight markers are depicted on the left. Data are representative of two independent experiments.
shown that the proteasome is responsible for E2-mediated ER degradation (6–9). However, mammalian cells contain hundreds of E3 ubiquitin ligases, each of which binds to a specific protein substrate that has been targeted for degradation (39), and the E3 ligase(s) responsible for destruction of ER had not been identified previously. In this study we document that E6AP significantly reduced ER levels in MCF-7 cells. Enzymatic activity is necessary for ER degradation because a catalytically inactive mutant form of E6AP (E6AP C883A) did not alter the amount of ER, despite a documented ability to bind ER. Endogenous E6AP specifically coimmunoprecipitated with ER from cell lysates and ER bound to GST–E6AP. The binding appears to be specific for E6AP as Nedd4, another HECT domain E3 ligase that is homologous to E6AP in its C terminus (30), neither coimmunoprecipitated with ER nor induced ER degradation. Collectively, these data reveal that E6AP is a component of a degradation pathway responsible for ER proteolysis.

E2-mediated down-regulation of ER has been linked to its transcriptional activity (6, 10). For example, the p160 coactivator AIB1 both recruits transcription factors involved in ER gene activation and mediates ER degradation by E2 (10). Calsmodulin also has a dual role in regulating ER by affecting both transcription and degradation. However, the effects of calmodulin are different from those of AIB1. Calmodulin binding is necessary for E2-stimulated transcription (16, 22) and blocks ER degradation. Our findings provide a molecular mechanism to explain the prior observations that calmodulin is overexpressed in breast cancer (31) and that calmodulin antagonists reduce the growth of breast cancer cells (41, 42) and synergistically augment anti-estrogen breast cancer (31) and that calmodulin antagonists reduce the growth of HPV16 oncocites. These findings raise the question as to how E2 and HPV16 interact to induce carcinogenesis. It is tempting to speculate that the interaction of E6AP with ER identified in this study may contribute to this process. Further work is necessary to test this provocative hypothesis.

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